REMARKS

Claims 1-21 are pending in this application. Amendments to claims 1, 7, 11 and 13 are provided with this Response.

Claim Rejections under 35 USC 102

The Office action of 06/02/2005 states that claims 1-3 and 5-21 stand rejected under 35 USC 102(b) as being anticipated by Kaleko (WO/97/25446). On page 4 of that Office action, it is stated that ". . . it is maintained that the language of the instant claims does not exclude the presence of the elements as a terminal protein attached to or associated with an ITR. There is no language in the instant claims reciting that a terminal protein is not attached to an ITR, and the claim language is open. Therefore, the rejection is maintained."

Without conceding to the correctness of this rejection, to advance the claims to allowance, Applicant amends all independent claims, claims 1, 7, 11 and 13, to include exclusionary language that states that "an adenovirus encoded terminal protein is not linked to the first or to the said second nucleic acid sequence." This phrase excludes the possibility that an adenovirus vector is formed in accordance with the claims where that vector includes as an element of the vector an adenovirus encoded terminal protein attached to or associated with an ITR.

This phrase is supported in the specification as follows. First, in two paragraphs in the Summary, from page 4, line 24 to page 6, line 6, two embodiments are distinguished, and only the latter one is stated to comprise a DNA-Terminal Protein ("TP") complex. This is further supported on page 17, line 26, where it is stated, "In a preferred embodiment of the present invention, a system is described for the construction of novel Ad vectors, or alteration of existing Ad vectors, by the use of a site-specific recombinase." This is immediately followed on page 18, line 1, with "In a *further* embodiment of the invention, an infectious viral DNA-TP complex is

engineered to take advantage of recombinase-mediated site-specific recombination and the enhanced level of infectivity achieved through the presence of the terminal protein." (italics emphasis added) These two parts of the specification make clear that the first of the each set of disclosed embodiments does not comprise DNA-TP.

Further support of this point is from page 5, line 15 through page 6, line 6, where it is explained that "In a further embodiment of this invention, DNA-TP complexes are utilized . . .". An example of this embodiment is later provided as Example 10, page 45, as supported by figures 12 and 13 (also see FIGs. 8b and 8c). Thus, although the use of DNA-TP complexes was articulated, by the logic of dichotomy, in other embodiments there are no DNA-TP complexes. Key examples where there is no TP associated with DNA are Examples 1 and 6. It is appreciated that for the examples in which first and the second nucleic acid sequences are in the form of circularized plasmids, it is not structurally possible to have TP associated with the ITR in such construct. Accordingly, in summary, the added exclusionary language is supported by the existence of such embodiments and as the different embodiments are described and distinguished in the specification. Based on the amendments herein, the claims are limited to embodiments of the invention that do not comprise a DNA-TP complex in an infectious adenovirus vector that is produced in accordance with these claims. Thus, these amendments are supported and do not add new matter.

Further as to the role of terminal protein (TP) in adenoviral replication, during the telephone interview on 28 July 2005, the Attorney for Applicant indicated that he would attempt to provide supporting material and/or explanation for the role of TP. Accordingly, provided herein are pages 671 to 682 of a review article by Challberg and Kelly, entitled Animal Virus DNA Replication (Annu. Rev. Biochem. 1989. 58:671-717). Further, as supported by this review article, it is noted that adenovirus TP is synthesized in the form of a larger 80-kd precursor (pTP), and the pTP is active in initiation, such as during repeated replication of copies of the original infecting DNA template (see page 675 and 678-680). Although the TP may be produced

in a host cell, the terminal protein precursor (pTP) is one of three replication-related proteins encoded by genes in the adenovirus genome (see page 674). During packaging of the viral genome into virions, pTP undergoes proteolysis that results in the formation of the 55-kd TP (p. 674). Thus, it is appropriate to state that TP associated with adenoviral DNA is adenoviral encoded (this also is supported in the specification on page 37, lines 3-5, recognizing, however, that the embodiment referred to is excluded in the claims as presented herein). The 55-kd TP is associated with the adenoviral DNA in the virion, and appears to remain so associated to the original, infecting copy of adenoviral DNA during an infection of a cell by such virion. Also, while the 55-kd TP attached to adenoviral DNA does provide an efficient template for initiation of adenoviral replication (p. 678), this is not necessary. Further explanation of the use of TP is found in the specification, from page 5, line 15 through page 6, line 6.

Also, as additional information about adenoviral DNA complexed with terminal protein, two early references are provided; these were referenced on page 6, lines 23-26, of the specification, and are the Sharp et al. article and the Chinnadurai et al. article.

Returning to the significance of the discussion of TP in the Kaleko reference, first, it is appreciated that Kaleko neither suggests nor provides a motivation for one to practice his invention without the use of TP on at least one of the polynucleotide sequences. Second, Kaleko consistently refers to the presence of TP, such as would be obtained by a more tedious extraction process from adenoviral DNA from virions, so when referring to TP Kaleko apparently is not considering nor concerned with the normal roles of pTP upon DNA copies after infective entry of a recombinant adenoviral vector into a cell. Third, because Kaleko does not teach, suggest, nor provide a motivation to combine teachings to practice recombinase-based recombination without TP attached to at least one polynucleotide used to produce an infectious adenovirus, Kaleko does not enable the present invention as it is presently claimed.

Based on the above, Kaleko does not anticipate the claims as amended, nor can render it obvious,

and Applicant respectfully requests reconsideration and withdrawal of the above-indicated basis for claim rejection.

Claim Rejections under 35 USC 103

The Office action of 06/02/2005 states that claim 4 stands rejected under 35 USC 103(a) as being unpatentable over Kaleko (WO/97/25446) in view of Bett et al. (Proc. Natl. Acad. Sci. USA 91:8802-8806 (1994)). The rejection refers to the reasoning provided with regard to the above-referenced anticipation rejection.

The same arguments and distinctions as provided above for the anticipation rejection of claim 1-3 and 5-21 apply to this obviousness rejection of claim 4. In particular, because Kaleko does not teach, nor suggest nor provide a motivation to practice the recombinase-based recombination to form an infectious adenovirus vector without TP, it is not proper to use this reference in an obviousness rejection for claims that explicitly state that "the terminal protein is not linked to the first or to the said second nucleic acid sequence."

Accordingly, Applicant respectfully requests reconsideration and withdrawal of the above-indicated basis for claim rejection.

Double Patenting Rejection

Claims 1, 7, 11 and 13 stand rejected under the judicially created doctrine of obviousness-type double patenting, as being unpatentable over claims 1-12 of U.S. Patent No. 6,379,943. Without conceding to the correctness of this rejection, Applicant herewith provides a Terminal Disclaimer to overcome this basis of rejection.

Accordingly, Applicant respectfully requests reconsideration and withdrawal of the above-indicated basis for claim rejection.

Incorporation of Tables from Parent

In studying the specification in order to prepare this Reply, Attorney for Applicant has come to the conclusion that Tables 1-5, present in the parent application, apparently were inadvertently omitted from the filing papers of the present application. To rectify this oversight, Applicant provides the Tables 1-5 from the parent, Serial number 09/263,650, filed March 5, 1999, now U.S. Patent No. 6,379,943. Incorporation of these tables is further supported by the incorporation by reference statement in the present application, found on page 18, lines 23-25, and the first paragraph cross reference to the noted parent.

Entry of Tables 1-5 to the application is respectfully requested.

Applicant respectfully requests that a timely Notice of Allowance be issued in this case.

* * * * * * *

The Examiner is invited to call the undersigned if clarification is needed on any aspects of this Reply/Amendment, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

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Table 1. Cotransfections on 293 and 293Cre cells for rescue of LacZ (± loxP)

Plasmid combo	µg DNA	Plaques/dish (293 cells)	(Totals)	Plaques/dish (293Cre cells)	(Totals)
pCA36:pBHG10	5:5	0, 0, 0, 0		0, 1, 2, 0	
	5:10	0, 0, 0, 1		1, 0, 0, 0	
	10:10	2, 0, 1, 1		1, 2, 0, 0	
			(5)		(7)
pCA36:pBHG10lox	5:5	0, 0, 0, 1		0, 0, 0, 0	
	5:10	0, 0, 0, 1		0, 0, 0, 0	
	10:10	0, 0, 2, 1		0, 0, 0, 0	
			(5)		(0)
pCA36lox:pBHG10	5:5	1, 3, 1, 0		0, 1, 0, 1	
	5:10	0, 1, 0, 0		0, 0, 1, 2	
	10:10	0, 0, 0, 0		0, 1, 1, 0	
			(6)		(7)
pCA36lox:pBHG10lox	5:5	1, 0, 0, 1		15, 14, 20, 20	
	5:10	0, 0, 0, 0		11, 15, 12, 16	
	10:10	0, 0, 1, 1		18, 9, 10, 8	
			(4)		(168)

Table 2. Cotransfections on 293 and 293Cre cells for rescue of LacZ (± loxP)

Plasmid combo	µg DNA	Plaques/dish (293 cells)	(Totals)	Plaques/dish (293Cre cells)	(Totals)
pCA36:pBHG10lox	5:5	1, 1, 2, 6, 2, 3	(15)	1, 1, 2, 1, 2, 3	(10)
pCA36lox:pBHG10lox	5:5	1, 2, 2, 2, 2, 1	(10)	41,44,41,41,44,31	(242)
pCA36loxΔ:pBHG10lox	5:5	0, 0, 0, 0, 0, 0	(0)	41,36,55,34,24,40	(230)
FG140	1	72, 72		150, 115	

Table 3 Efficiency of Ad vector rescue by cotransfection with pBHG10lox and various shuttle plasmids[a]

Cell line	Shuttle plasmid	Plaques/dish	Average/dish
293	pCA36lox	6, 2, 3, 3, 5	3.8
	pCA36loxΔ	1, 4, 0, 0, 0	1.0
	pCA36loxΔCreR	2, 2, 4, 3, 2	2.6
	pCA36loxΔCreT	9, 4, 4, 7, 3	5.4
293Cre	pCA36loxΔ	23, 28, 22, 28	25.3

[a] 5μg of all plasmids were used in cotransfections.

Table 4. Efficiency of Ad vector rescue by cotransfection of 293 cells with pBHG10lox and shuttle plasmids encoding Cre[a].

Cell line	Shuttle plasmid	Plaques/dish	Average/dish
293	pCA36lox	2, 3, 1, 0, 1	1.4
	pCA36loxΔ	1, 0, 0, 0, 0	0.2
	pCA36loxΔCreT[b]	3, 1, 5, 2, 4	3.0
	pCA36loxCreITR[b]	21, 20, 42, 34, 40	31.4

[a]All cotransfections performed with 5 μg of the indicated shuttle plasmid and 5μg of pBHG10lox

[b]Plasmids illustrated in figure 8c.

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ANIMAL VIRUS DNA REPLICATION[1]

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CONTENTS

PERSPECTIVES AND SUMMARY

Much of the impetus for studying the replication of animal virus genomes comes from a desire to understand the events that occur during the replication of eukaryotic chromosomes. Viruses offer many advantages for the study of

eukaryotic DNA replication. Viral genomes are relatively simple and can be readily manipulated by modern genetic methods. In addition, the replication of some viral genomes has proven amenable to analysis in cell-free systems. These facts significantly enhance the ability to analyze replication mechanisms at the molecular level. There are a number of potentially useful viral systems, and this review focuses on four of the best characterized: (*a*) adenovirus, (*b*) SV40, (*c*) herpes simplex virus, and (*d*) bovine papillomavirus. Each system has certain unique virtues that can be exploited to gain insight into different aspects of the replication process.

Adenovirus DNA replication occurs by a process that is significantly less complex than chromosomal DNA replication. Replication initiates by a novel protein priming mechanism, and all daughter strands are elongated by a continuous mode of synthesis such as occurs at the leading strand of a chromosomal replication fork. The biochemical dissection of a soluble in vitro system capable of faithfully replicating adenovirus DNA has led to the identification of most of the proteins involved. Adenovirus DNA replication requires the participation both of virus-encoded replication proteins and host-cell–encoded transcription factors. Since a linkage between replication and transcription has now been observed in other systems, it is likely that further analysis of the adenovirus system will provide insights that are of general importance.

The SV40 genome represents a more complete model system for studying cellular DNA replication. SV40 encodes only a single replication protein (T antigen) and relies predominantly on the host-cell replication machinery. In vivo studies have established that many of the details of SV40 DNA replication are closely similar to those of cellular DNA replication. Replication initiates at a fixed site on the viral genome and proceeds bidirectionally with continuous growth of leading strands and discontinuous growth of lagging strands. As in the case of adenovirus, an efficient cell-free replication system has been developed for SV40, and dissection of this system has identified several cellular replication proteins. A partial understanding of the mechanisms by which these proteins act is beginning to emerge, and it seems certain that this will be an area of continued rapid progress.

In contrast to SV40, herpes simplex virus (HSV) encodes many, if not all, of the proteins that are involved in the replication of its genome. Thus, HSV DNA replication has been studied by using a combination of genetics and biochemistry. The complete set of viral genes necessary for DNA synthesis has recently been identified, and the products of many of these genes have been purified and partially characterized. Several of these purified proteins have functions expected of replication proteins, including a DNA polymerase, a helicase, a primase, a single-stranded DNA binding protein, and an origin recognition protein. It seems likely that the availability of these purified

proteins will soon lead to the development of an in vitro system capable of specifically replicating HSV DNA. Genetic and biochemical dissection of such a system should provide important new insights into the molecular mechanisms of eukaryotic DNA replication.

Adenovirus, SV40, and HSV are all examples of viruses that normally multiply by productive cytocidal infection. In all of these cases, viral DNA replication begins soon after infection and continues at a high rate until the death of the host cell. In contrast, bovine papillomavirus (BPV) represents an example of a virus that is capable of multiplying as a stable extrachromosomal element. In this case, viral DNA replication is controlled so that the number of viral genomes doubles only once per cell cycle, and under normal circumstances the host is not killed. As in the case of SV40, the BPV genome is relatively small and encodes only a small number of proteins involved in DNA replication; viral DNA synthesis depends heavily on host-cell replication proteins. The biochemical analysis of BPV DNA replication is in its infancy, but genetic analyses have provided evidence for a negative control system that apparently ensures that each viral genome is replicated once and only once during each cell cycle. Bovine papillomavirus therefore represents an excellent model for illuminating mechanisms involved in regulating DNA replication.

ADENOVIRUS DNA REPLICATION

Adenovirus DNA replication is better understood than the replication of the other animal virus genomes because it was the first to be established in vitro (1). Although many of the molecular details remain to be worked out, the basic features of the adenovirus DNA replication pathway are now reasonably clear, and most of the proteins involved in the replication process have been identified (2–5). On the basis of the information gathered to date, it is evident that the adenoviruses have evolved some very interesting and novel solutions to the replication problem, including the use of a "protein priming" mechanism for initiation and the diversion of cellular transcription factors to the viral replication machinery. It seems certain that further biochemical analysis of this system will illuminate and extend our understanding of DNA replication in the context of the animal cell.

The Adenovirus Chromosome

The genomes of the human adenoviruses are double-stranded linear DNA molecules containing approximately 35,000 base pairs. The 5' terminus of each strand of the viral genome is covalently attached to a virus-encoded protein (TP) with a molecular weight of about 55,000 (6, 7). In addition, the nucleotide sequences at the extreme ends of the genome are identical (8, 9).

Both of these structural features play important roles in the initiation of viral DNA replication.

Numerous in vivo studies have established that adenovirus DNA replication takes place in two stages (2–5). In the first stage, DNA synthesis is initiated at either terminus of the duplex viral genome by the protein priming mechanism (see below). The initiation process results in the establishment of a replication fork that moves from one end of the genome to the other. At each replication fork only one of the two parental DNA strands serves as a template for DNA synthesis. Thus, the products of the first stage of replication are a daughter duplex and a displaced single strand. In the second stage of DNA replication, the strand complementary to the displaced single strand is synthesized. It seems likely that the initial step in this process is the circularization of the single-stranded template by annealing of its self-complementary termini. The resulting duplex "panhandle" has the same structure as the terminus of the duplex adenovirus genome and is presumably recognized by the same initiation machinery that operates in the first stage of replication. Following a second initiation event, complementary strand synthesis proceeds from one end of the template to the other, generating a second daughter duplex. In both stages of adenovirus DNA replication there is only one priming event per nascent daughter strand, so all viral strands are synthesized in a continuous fashion from their 5' termini to their 3' termini.

The adenoviruses encode three proteins that play central roles in viral DNA replication: the terminal protein precursor (pTP), the adenovirus DNA polymerase (Ad pol), and the single-stranded DNA binding protein (DBP) (10–12). Together these three proteins consume approximately 25% of the coding capacity of the viral genome. The mRNAs for all three proteins are products of the same viral transcription unit and are produced by differential splicing of a common precursor (13). This genetic organization provides a simple mechanism for the coordinate regulation of the levels of replication proteins during infection. In addition to the virus-encoded proteins, adenovirus DNA replication requires the participation of several cellular proteins. Those identified to date include two cellular transcription factors (NF-I/CTF and NF-III/OTF-1) and a cellular topoisomerase activity (14–20). The biochemical roles of these viral and cellular replication proteins are discussed below.

Initiation of Adenovirus DNA Replication

THE PROTEIN PRIMING MODEL Initiation of adenovirus DNA replication occurs by a novel mechanism in which the first nucleotide in the new DNA chain becomes covalently linked to a virus-encoded protein, the terminal protein precursor. This mechanism is unique among the DNA viruses of mammals, but a similar mechanism operates during the replication of other

chromosomes, for example bacteriophage $\Phi 29$ (21). The protein priming model was first proposed following the discovery that the 5' ends of adenovirus DNA strands are covalently linked to the 55-kd terminal protein (22). Direct biochemical support for the model was obtained by analysis of the initiation reaction in the adenovirus cell-free replication system. Initial studies demonstrated that the adenovirus terminal protein is synthesized in the form of a larger 80-kd precursor (pTP), which is active in initiation (10, 13, 23, 24). The pTP is processed by proteolysis to the mature 55-kd form during packaging of the viral genome into virions (23). A series of isotope transfer experiments provided evidence that the critical first step in the replication reaction is the formation of an ester bond between the β-OH of a serine residue in the pTP and the α-phosphoryl group of dCMP, the first residue in the new DNA chain (10). The nascent strand then grows by extension from the 3' hydroxyl of the covalently bound dCMP residue (22). The subsequent development of a direct assay for the formation of a covalent complex between dCMP and the pTP (pTP-dCMP) made it possible to purify the pTP in functional form and to define the requirements for the initiation reaction (25–28). Work in a number of laboratories has shown that initiation is dependent upon the presence of specific nucleotide sequence elements at the termini of the viral genome and requires the participation of several viral and cellular proteins.

THE ADENOVIRUS ORIGIN OF DNA REPLICATION The natural template for adenovirus DNA replication in vivo or in vitro is the viral chromosome with the covalently attached terminal protein (TP or pTP). However, as first demonstrated by Tamanoi & Stillman (28), plasmids containing the cloned adenovirus terminal sequence will support initiation of DNA replication in vitro, provided that the plasmid is cleaved with a restriction enzyme in such a way that the adenovirus terminus is located near the end of the resulting linear DNA molecule. This observation provided definitive evidence that specific nucleotide sequence elements in the viral genome are recognized by the initiation machinery. The efficiency of initiation with plasmid templates is considerably lower than that observed with adenovirus chromosomes isolated from purified virions. Moreover, recent studies have revealed that the protein and cofactor requirements for initiation are somewhat different for the two templates (29–32). However, analysis of plasmids with deletion and/or base substitution mutations has been very useful for defining the nucleotide sequence requirements for initiation.

Most in vitro studies of the nucleotide sequence requirements for adenovirus DNA replication have been conducted with serotypes 2 or 5. Analysis of a large number of deletion and base substitution mutations has revealed that the adenovirus origin of DNA replication is complex, containing at least three

functionally distinct domains (17, 33–42). Domain A consists of the first 18 base pairs of the viral genome and represents the minimal origin of replication. The presence of domain A is absolutely required for initiation of adenovirus DNA replication, but templates containing only domain A initiate DNA synthesis at a very low efficiency. All adenovirus serotypes that have been examined share a common 10-base-pair sequence, ATAATATACC, within this region of the viral genome (34, 43, 44). It has been suggested that this conserved motif is important for the binding of the virus-encoded initiation proteins to the origin (32, 45). Domains B and C, while not absolutely required for initiation of adenovirus DNA replication, contribute significantly to the efficiency of the initiation reaction. Domain B consists of the segment between nucleotides 19 and 39 (36, 37, 40, 42). As in the case of domain A, there is considerable sequence conservation in this region among the various adenovirus serotypes. Many (but not all) adenovirus genomes contain a version of the consensus sequence TGG(A/C)NNNNNGCCAA. As described below, this motif is recognized by a cellular DNA-binding protein, nuclear factor I(CTF)(14–16). The presence of domain B increases the efficiency of initiation of adenovirus 5 DNA replication at least 10-fold. Domain C of the adenovirus origin includes nucleotides 40 to 51 and contributes an additional factor of three to the efficiency of initiation of viral DNA replication in vitro (17, 42). The consensus sequence, AT(G/T)N(A/T)AAT, has been identified in this region. A second cellular DNA-binding protein, nuclear factor III (ORP-C, OTF-1), recognizes this sequence (17–19). The spacing between the minimal origin and domain B appears to be critical for origin function (41, 42). The insertion or deletion of only a few base pairs between the two segments dramatically reduces the efficiency of initiation. This observation suggests that the initiation reaction may require relatively short-range interactions between the protein factors that bind to the various domains of the origin.

Analysis of the replication of deletion mutants in vivo is largely consistent with the general picture of the sequence organization of the adenovirus genome derived from the in vitro studies (46–51). Both the conserved sequence element in domain A and the nuclear factor I binding site in domain B have been shown to be essential for adenovirus 2 (or 5) DNA replication in cultured cells. The stimulatory effect of domain C has not yet been observed in vivo. In contrast to most adenovirus serotypes, the replication origin of adenovirus type 4 lacks a recognition site for nuclear factor I. It has been demonstrated that Ad4 DNA replication, both in vivo and in vitro, requires only the terminal 18 base pairs of the genome, which are identical to the minimal origin of Ad2 or Ad 5 (49, 52).

CELLULAR ORIGIN-BINDING PROTEINS Nuclear factor I (NF-I) was originally identified as a cellular factor that stimulated formation of pTP-dCMP

complexes by partially purified viral replication proteins (14). The stimulatory activity was subsequently purified to near homogeneity by recognition site affinity chromatography and shown to consist of a family of polypeptides with molecular weights between 52,000 and 66,000 (15). Recently, three human cDNA clones of NF-I have been isolated and characterized (53). The clones contain blocks of identical sequence interspersed with blocks of different sequence, suggesting that the corresponding mRNAs are generated by differential splicing. Analysis of the open reading frames in the clones suggests that each mRNA encodes a distinct protein. Thus, differential splicing may account, at least in part, for the multiplicity of NF-I polypeptides that have been observed. Although it has been demonstrated that the protein products of all three NF-I cDNA clones are active in stimulating adenovirus DNA replication in vitro, it is not yet clear whether all of the NF-I polypeptides are functionally equivalent (53).

The interaction between NF-I and its recognition sequence has been studied using chemical probes and in vitro mutagenesis (13, 39, 54). Taken together, these studies suggest that the optimal recognition site consists of the symmetrical sequence $TTGGCN_5GCC(A/A)$. The principal contacts between protein and DNA are in the major groove, and nearly all of the contacts are accessible from one side of the helix (54). Given the symmetry of the protein-DNA contacts, it seems likely that NF-I binds as a dimer.

Purified nuclear factor I stimulates initiation of adenovirus DNA replication in vitro at least 30-fold (15, 42). The binding of NF-I to its recognition sequence is essential for the stimulatory effect, since base substitution mutations in the viral origin that abolish binding greatly reduce the efficiency of initiation (18, 36, 37, 39, 40, 42). The precise role of NF-I in the initiation reaction is not yet clear (see below).

Nuclear factor III (ORP-C) was identified as a stimulatory factor that recognized the sequence element TATGATAAT within domain C of the adenovirus 2 origin of replication (17, 18). The factor has been purified to homogeneity by recognition site affinity chromatography and shown to consist of a 92-kd polypeptide (55). Experiments with various chemical probes indicate that the protein makes both major and minor groove contacts with the DNA and that the contacts are not confined to one side of the helix (56). The binding site for NF-III is very close to that for NF-I; both proteins contact the same A/T base pair at position 39 in the adenovirus origin of replication (56). Despite their proximity, there is no evidence for cooperativity in binding (18, 56). Both NF-I and NF-III are required for optimal levels of DNA replication in vitro (17, 18, 55, 56), but the requirement for NF-III in vivo has not yet been demonstrated.

Interestingly, both nuclear factor I and nuclear factor III also appear to function as cellular transcription factors. A number of viral and cellular promoters contain functionally significant sequence elements that are related

to the NF-I recognition sequence (57–67). A protein factor (CCAAT transcription factor or CTF) that recognizes the GCCAAT motif present in several such promoters has been purified to homogeneity and shown to be capable of stimulating transcription of the human α globin gene in vitro and in vivo (68). Purified CTF was found to consist of a series of polypeptides with molecular weights similar to those previously described for NF-I (15). Detailed comparison of the physical and biochemical properties of CTF with those of NF-I demonstrated that the two groups of proteins are indeed identical (16).

The recognition site for nuclear factor III is similar to the octamer sequence that has been implicated in the transcriptional regulation of several genes, including the histone H2b, immunoglobulin, and small nuclear RNA (U1 and U2) genes (17, 18). Binding studies have demonstrated that NF-III binds to the promoter/enhancer regions of several such genes (69). A 92-kd protein factor (octamer transcription factor or OTF-1) that recognizes the octamer sequence in the histone H2b promoter and markedly stimulates H2b transcription in vitro has been purified from HeLa cells (70). The purified OTF-1 protein is physically and biologically indistinguishable from NF-III (19). The implications of the finding that cellular sequence-specific DNA-binding proteins can participate in both transcription and DNA replication are not yet clear. On the one hand, adenovirus may have simply subverted cellular transcriptional factors for its own purposes. On the other hand, there is abundant circumstantial evidence for a fundamental relationship between transcription and replication in eukaryotic cells (5, 71–73). Indeed, several examples of transcriptional signals that significantly affect the efficiency of DNA replication are documented in other sections of this review. Further study of the roles of NF-I and NF-III in adenovirus DNA replication may lead to better understanding of the mechanistic role of transcriptional factors in DNA replication.

REQUIREMENTS FOR THE INITIATION REACTION Initiation of adenovirus DNA replication is assayed in vitro by measuring the formation of a covalent complex between dCMP and the 80-kd pTP (25–28). The initiation reaction is absolutely dependent upon the presence of a DNA template. The most efficient template is the adenovirus chromosome containing the covalently attached 55-kd terminal protein, although other DNA molecules, such as linear plasmids or single-stranded DNA molecules, will support pTP-dCMP complex formation to some extent (see below). With adenovirus chromosomes as template, optimal initiation requires a minimum of four proteins: two cellular proteins, NF-I and NF-III (14, 15, 17, 18), and two virus-encoded proteins, pTP and the 140-kd adenovirus DNA polymerase (11, 74–76). The viral proteins copurify through several chromatographic steps,

and their sedimentation behavior suggests that they exist in a 1:1 complex (11, 74, 75). The complex can be separated into the individual polypeptides by glycerol gradient sedimentation in the presence of urea (74, 75, 77). This has made it possible to demonstrate the absolute requirement for both the pTP and the DNA polymerase in the initiation reaction (74, 75, 77). Initiation also requires ATP, which appears to be serving an effector function, since nonhydrolyzable analogues also stimulate initiation, and no ATP hydrolysis has been detected (32). It has been reported that a third virus-encoded protein, the 72-kd single-stranded DNA-binding protein (DBP) (78), stimulates initiation several-fold, but the protein is clearly not an essential participant in the reaction (18, 32).

As described above, duplex templates that lack the covalently attached terminal protein (e.g. linear plasmids containing the adenovirus origin of DNA replication at one terminus) are capable of supporting the initiation reaction, albeit at lower efficiency than adenovirus chromosomes. With such templates, an additional protein, factor pL, is required for efficient formation of pTP-dCMP complexes (29, 30). Factor pL has been purified to near homogeneity and shown to be a 44-kd polypeptide with 5' → 3' exonuclease activity (30). The pL exonuclease appears to activate adenovirus templates that lack the terminal protein by degrading the 5' end of the DNA strand that is normally displaced during adenovirus DNA replication (30, 31). This creates a short single-stranded region at the 3' end of the DNA strand that normally serves as the template for adenovirus DNA synthesis. Similar partially single-stranded templates, constructed using synthetic oligonucleotides, support the initiation reaction in the absence of factor pL (31). Thus, the presence of a single-stranded region at the 3' end of the template strand appears to allow the system to bypass the requirement for the 55-kd terminal protein on the input DNA. One possible interpretation of this result is that the 55-kd terminal protein attached to adenovirus chromosomes plays some role in opening the duplex during the early stages of the initiation process (see below). Except for the requirement for factor pL, the protein requirements for initiation on partially single-stranded templates are the same as those for adenovirus chromosomes (32). However, ATP is no longer required (32).

Single-stranded DNA molecules will also support formation of pTP-dCMP complexes in vitro (28, 29, 33, 35, 79). The sequence requirements for initiation with single-stranded templates appear to be somewhat less stringent than with adenovirus chromosomes or plasmid DNAs (35). A large number of single-stranded DNA molecules, including those lacking the specific adenovirus origin sequences, have been observed to support pTP-dCMP complex formation with varying degrees of efficiency. However, it has been reported that an oligonucleotide containing the template strand of the adenovirus origin is 5–20 times as active in initiation as other single-stranded DNA molecules

(32, 80). With single-stranded DNA templates, initiation is dependent on the virus-encoded replication proteins, but is not dependent on NF-I or NF-III (32). Both ATP and the adenovirus DBP are inhibitory.

MECHANISM OF INITIATION The precise order of events during initiation of adenovirus DNA replication is not yet clear. The finding that single-stranded or partially single-stranded templates can support pTP-dCMP complex formation suggests that initiation is a two-step process (Figure 1). In the first step, the terminal region of the viral genome is unwound, exposing a short single-stranded region. In the second step, a dCMP residue is covalently linked to the pTP. Elucidation of the protein requirements for localized unwinding at the adenovirus origin must await the development of a direct unwinding assay. However, the available data suggest that the 55-kd terminal protein attached to the template DNA may play a role in unwinding, since the requirement for the terminal protein is obviated by presence of a single-stranded region at the end of the template. It is also possible that NF-I, NF-III, pTP, or the adenovirus polymerase participate in strand opening within the origin. The binding of the pTP and adenovirus DNA polymerase to the template strand of the origin presumably takes place following (or perhaps during) the unwinding reaction. There is some evidence that the complex of pTP and Ad polymerase may interact with sequence elements in the origin, but these interactions must be of relatively low specificity. It is possible that one role of NF-I and NF-III is to facilitate the binding or positioning of the pTP and adenovirus DNA polymerase on the DNA template. The final step in initiation, the formation of the covalent pTP-dCMP complex, probably occurs once the pTP-Ad pol complex is correctly positioned on the exposed template strand. Although both the pTP and the adenovirus DNA polymerase are required for the initiation reaction, an important unresolved question is whether the adenovirus DNA polymerase catalyzes the transfer of dCMP to the pTP or whether this is an autocatalytic process.

Elongation of Nascent DNA Chains

The synthesis of full-length adenovirus DNA strands in vitro requires the pTP, the Ad DNA polymerase, the Ad DBP, and nuclear factors I–III. At present there is good evidence that three of these proteins (Ad DNA polymerase, Ad DBP, and nuclear factor II) are directly involved in chain elongation (20, 81, 82). Although there is no direct evidence for the involvement of the other adenovirus replication proteins in elongation, this possibility cannot be completely ruled out. The adenovirus DNA polymerase is a 140-kd protein with physical and biochemical properties distinct from the other known eukaryotic DNA polymerases (11, 74, 81, 82). The enzyme is capable of utilizing a variety of deoxyribonucleotide homopolymer template-primers,

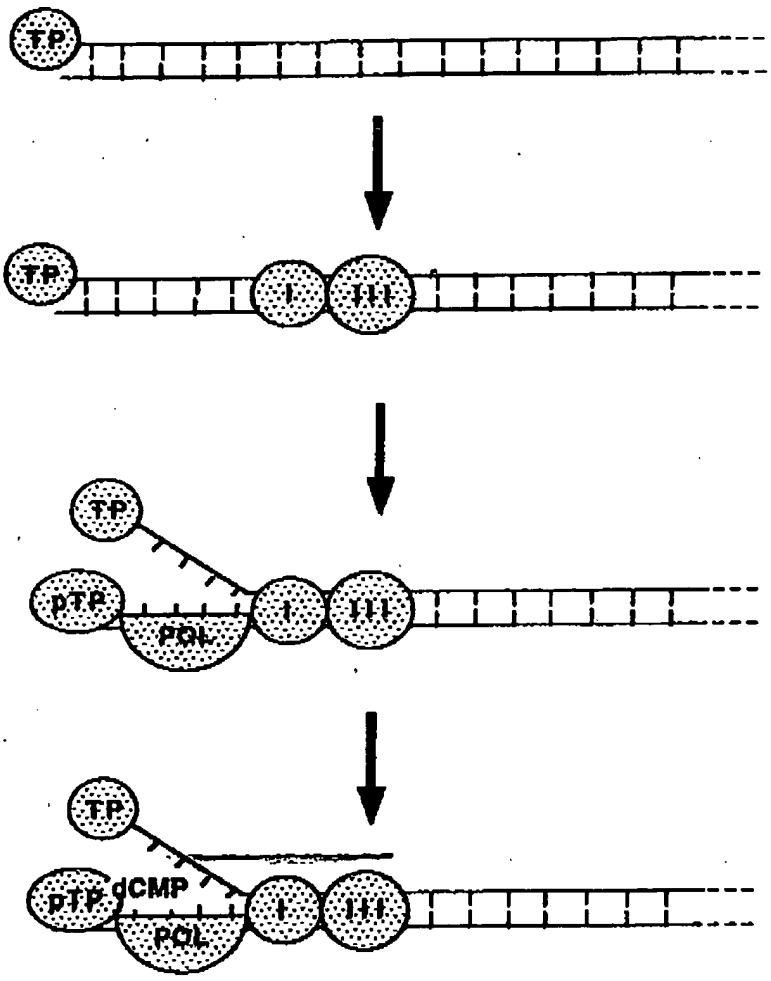

Figure 1 Diagrammatic representation of the initiation of adenovirus DNA replication. See text for details. Abbreviations used: I, nuclear factor I; III, nuclear factor III; TP, terminal protein; pTP, preterminal protein; POL, adenovirus DNA polymerase.

but is relatively inactive with RNA primers (82). Polymerase activity is inhibited by dideoxynucleotides and is resistant to aphidicolin. The purified polymerase contains an intrinsic 3'→5' exonuclease activity that is specific for single-stranded DNA and probably serves a proofreading function during polymerization (82). The Ad DBP is a 59-kd phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72,000 (12, 83). The DBP binds tightly and cooperatively to single-stranded DNA in

sequence-independent fashion (12, 83–87). At saturation approximately one molecule of DBP is bound for every seven nucleotides (85). With poly(dT) as template and oligo(dA) as primer the DBP stimulates DNA synthesis by the Ad DNA polymerase as much as 100-fold (82). The stimulatory effect is quite specific, since the *Escherichia coli* SSB has no effect on Ad DNA polymerase activity and the Ad DBP does not stimulate the activity of other DNA polymerases such as HeLa DNA polymerase α. Based upon these and other results (81, 82), it seems likely that there is a highly specific interaction between the DBP and the Ad DNA polymerase that increases the efficiency of polymerization; however, a stable complex of the two proteins has not yet been detected. In the presence of the DBP, the Ad polymerase is a highly processive enzyme, capable of synthesizing DNA chains at least 30,000 nucleotides in length from a single primer terminus (82). Moreover, under these conditions the polymerase appears to be able to translocate through long stretches of duplex DNA (81). Thus, it is possible that fork movement during adenovirus DNA replication does not require a separate helicase activity. Rather, unwinding of the parental strands may be mediated solely by the Ad DNA polymerase and DBP, and the energy required for unwinding may be provided by the hydrolysis of deoxynucleoside triphosphates. This possibility is consistent with the observation that little, if any, ATP hydrolysis occurs during adenovirus DNA replication in vitro (81).

In the presence of the pTP, the adenovirus DNA polymerase, the DBP and NF-I nascent adenovirus DNA chains are elongated to only about 25% of full length (20). Synthesis of complete adenovirus DNA strands requires an additional cellular protein, nuclear factor II (20). Nuclear factor II from HeLa cells has a native molecular weight of approximately 30,000 and copurifies with a DNA topoisomerase activity. Human or calf thymus topoisomerase I (but not *E. coli* topoisomerase I) will substitute for nuclear factor II in the adenovirus DNA replication reaction. The precise function of nuclear factor II in adenovirus DNA chain elongation is not yet clear. Since the protein has no significant effect on the synthesis of nascent strands up to 9 kb in length, it is presumably required to overcome the inhibitory effects of some DNA structure that appears only after extensive DNA synthesis.

SV40 DNA REPLICATION

SV40 has proven to be an excellent model system for studying the mechanisms of cellular DNA replication (88–91). The viral genome consists of a circular duplex DNA molecule of about 5000 base pairs and contains one origin of DNA replication. SV40 DNA replication takes place in the nucleus of the host cell where the viral genome is complexed with histones to form a nucleoprotein structure (minichromosome) indistinguishable from cellular

chromatin. Since SV40 encodes only a single replication protein (T antigen), the virus makes extensive use of the cellular replication machinery. As a result there are many similarities between viral and cellular DNA replication. In both cases initiation of DNA synthesis results in the establishment of two replication forks that move in opposite directions. At each fork one of the two nascent strands (the leading strand) grows continuously, while the other strand (the lagging strand) grows discontinuously by joining together small (ca. 200 bp) segments of DNA that are independently initiated with RNA primers. Completion of replication occurs when two oppositely moving forks meet. In linear cellular chromosomes the two merging forks originate from adjacent origins, while in circular SV40 chromosomes they have a single origin.

Much has been learned about SV40 DNA replication from in vivo studies (see reviews in Refs. 88–91). However, the recent development of an efficient cell-free replication system has greatly accelerated progress in understanding the molecular mechanisms involved (92). An important dividend of the dissection of the cell-free system has been the identification and functional characterization of components of the cellular replication apparatus. Thus, this review focuses on in vitro studies.

Initiation of SV40 DNA Replication

THE ORIGIN OF DNA REPLICATION The SV40 origin of replication is a 64-base-pair segment of the viral genome that contains all of the nucleotide sequence elements that are required for initiation of viral DNA replication in vitro and in vivo (93–106). Careful genetic analysis of base substitution mutations has revealed that the origin is complex, consisting of at least three functionally distinct sequence domains (104–106). At the center of the origin are four copies of a pentomeric sequence motif (GAGGC) organized as an inverted repeat. This sequence element is recognized by the viral initiation protein, T antigen (101, 106–120). On one side of the T antigen–binding site is a 17-base-pair segment containing A/T base pairs (105). It is suspected that this is the initial site of strand opening during initiation of SV40 DNA replication. On the other side of the T antigen–binding site is a 15-base-pair imperfect palindrome of unknown function. All three sequence domains of the origin are required for SV40 DNA replication, and there is some evidence that the spacing between them is critical for origin function (104).

Although the 65-base-pair core origin region is sufficient to support the initiation of SV40 DNA replication, sequences outside of the core can significantly influence the efficiency of initiation. A second T antigen–binding site located adjacent to the core origin increases replication efficiency several-fold both in vivo and in vitro (97, 101–103, 121, 122). Of even greater importance are elements previously associated with the activation of SV40 transcription,

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The Infectivity of Adenovirus 5 DNA–Protein Complex

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Adenovirus 5 DNA–protein complexes were prepared by treating virions with 4 *M* guanidinium chloride and resolving the faster sedimenting viral DNA from released capsid protein. These complexes were characterized by both gel electrophoresis and electron microscopy. After dialysis into saline buffer, approximately one-half of the viral DNA–protein complex was aggregated by association at its termini into conformations other than linear monomer length duplex molecules. However, the monomer length linear molecules in the viral DNA–protein complex sample also have protein attached to both of their termini: These linear molecules are resistant to digestion by a processive nuclease, *adenosine triphosphate-dependent deoxyribonuclease*, that requires a free terminus for activity, while Pronase-treated adenovirus DNA is susceptible to digestion, and the restriction endonuclease cleavage fragments from both ends of the viral DNA–protein complex migrate at an anomalous rate during electrophoresis in an agarose gel. As the infectivity of Ad5 DNA is exceptionally low, the infectivity of the DNA-protein complex was tested using the calcium technique for transfection. The efficiency of transfection with the Ad5 DNA–protein complex is about 100-fold higher than that of Pronase-treated Ad5 DNA.

INTRODUCTION

The study of the biochemistry of the adenovirus genome began with the efforts of Green and his co-workers in the early 1960s (Green, 1970). Adenovirus DNA, extracted by disruption of virions by detergent and digestion with Pronase, was characterized as a duplex molecule with a sedimentation velocity coefficient of 81 S which could be denatured to give intact complementary single strands. Later, with the advent of techniques to visualize duplex DNA, the length of the linear duplex DNA genome was measured directly, yielding a molecular weight of 23×10^6 daltons (Green *et al.*, 1967). When sophisticated techniques for the visualization of single-strand DNA in the electron microscope became available, denatured adenovirus DNA was seen to form single-strand circles (Garon *et al.*, 1972; Wolfson and Dressler, 1972). Near the terminus of each strand was a sequence of bases between 70 and 140 bases long that was complementary to sequences adjacent to the other terminus (Roberts *et al.*, 1974): The single-strand circles were formed by the annealing of these homologous regions.

The structure of the termini of the viral genome became more novel when Robinson *et al.* (1973) reported that if adenovirus DNA was released from virions by treatment with 4 *M* guanidinium chloride and the extended duplex DNA was visualized in the electron microscope, the termini of a large fraction of the unit length DNA were aggregated. The association of the termini of one molecule resulted in a circle; the association of a circle with the terminus of a linear molecule formed a lariat structure; the association of four termini of two duplex yielded either a dimer length circle or figure 8. All of these structures, as well as many more complex forms, were observed (Robinson *et al.*, 1973). Treatment

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of samples of viral DNA with Pronase before mounting for visualization in the electron microscope eliminated all the complicated structures and only linear DNA was observed. Robinson *et al.* (1973) proposed that a virion protein, firmly attached at the termini of the DNA, was responsible for the intra- and intermolecular association.

The phenomenon of transfection occurs with about the same efficiency in mammalian cells as it does in bacteria. For example, several protocols give an efficiency of 10^3 PFU/μg when SV40 DNA is used to transfect monkey cells in culture (Pagano *et al.*, 1967; Graham and Van der Eb, 1973 a,b). Similarly, Herpes simplex DNA, approximately 80 times larger than SV40, has an efficiency of infectivity of 1000 PFU/μg (Graham *et al.*, 1973; Sheldrick *et al.*, 1973). In comparison with these efficiencies of transfection by other viral DNAs, adenovirus DNA has low infectivity. The highest reported efficiency of infection b adenovirus DNA is 20 PFU/μg or approximately 10^{-4} that expected from the SV40 DNA efficiency (Graham and Van der Eb, 1973a,b). This low infectivity of adenovirus DNA suggests that viral DNA alone is not sufficient for initiating the lytic cycle. Thus, one or more virion protsins might be necessary to efficiently commence infection. These proteins could act in a general role, for example, by altering the physiology of the cell, or in a more specific fashion by interacting with the viral DNA to alter its expression or stability following infection. With this in mind, we have prepared adenovirus 5 (Ad5) DNA–protein complexes using a slight modification of the protocol of Robinson *et al.* (1973) and tested its efficiency of transfection. In addition, a preliminary characterization of some of the properties of this Ad5 DNA–protein complex is reported.

MATERIALS AND METHODS

Cells. HeLa cells were grown either in suspension cultures containing minimal essential medium (Joklik modified), purchased from GIBCO and supplemented with 5% horse serum, or in monolayer (obtained from J. F. Williams) cultures containing Dulbecco's modification of Eagle's medium (D medium) supplemented with 10% calf serum (Dulbecco and Freeman, 1959).

Viruses. Adenovirus 5 seed stock was obtained from J. F. Williams and new seed stocks were prepared by propagation in suspension HeLa cells (Pettersson *et al.*, 1967). Virus titers were determined by plaque formation on monolayer HeLa cells (Williams, 1970). ^{32}P-labeled Ad5 was prepared by the addition of 2 mCi of $H_3{}^{32}PO_4$ to 500 ml of 5×10^5 cells/ml immediately after infection. These cells were grown in phosphate-free medium supplemented with 2% horse serum and virus was harvested 48 hr after infection. Ad5 virus was purified by the method of Green and Pina (1963) as modified by Lonberg-Holm and Philipson (1969).

DNA. Ad5 DNA was released from virions in 0.01 *M* Tris, pH 7.9, and 0.01 *M* EDTA, pH 7.9, by digestion with 20 μg/ml of Pronase in the presence of 0.5% SDS for 0.5 hr at 37°. Following digestion, the sample was adjusted to 0.1 *M* NaCl and extracted twice with phenol saturated with 1.0 *M* Tris, pH 8.5. To remove the remaining phenol, the DNA samples were dialyzed at 4° against three changes of buffer of 0.02 *M* Tris, pH 8.5, and 0.002 *M* EDTA, pH 8.6. The DNA concentration was determined from the optical density at 260 nm assuming 1 OD unit is equivalent to 50 μg/ml.

Gel electrophoresis. Agarose gels were prepared and run as described by Sharp *et al.* (1973) except that the electrophoresis buffer was modified to 0.04 *M* Tris, 0.05 *M* $NaC_2H_3O_2$, 0.001 *M* EDTA, pH 8.3. All gels were stained by immersion in the electrophoresis buffer containing 0.5 μg/ml of ethidium bromide, and the fluorescence of the bound dye was photographed as described in Sharp *et al.* (1973). Gels containing ^{32}P-labeled DNA were sliced into 1-mm sections and Cerenkov radiation was counted in a scintillation counter.

Enzymes. Endonuclease Eco R1 was prepared from *E. coli* strain RY-13 (Yoshimori, 1971) as described by Gallimore *et al.* (1974). Pronase was purchased from Calbiochem and dissolved in 0.01 *M* Tris, pH

7.9, 0.01 *M* EDTA, pH 7.9, to make a 20-mg/ml stock solution. This solution was autodigested for 3 hr at 37°.

Transfection. The protocols for transfection using DEAE-Dextran or $(Ca)_3(PO_4)_2$ are slight adaptations of those of Pagano *et al.* (1967) and Graham and Van der Eb (1973a,b), respectively. More explicitly, for both procedures, monolayer cells were suspended by trypsin digestion and diluted to 10^6 cells per 6-cm-diameter dish 24 hr before transfection. After transfection, cells were maintained at either 37° for 10 days or 38.5° for 7 days before staining with neutral red (Williams, 1970). The monolayers were overlayed with 5 ml of fresh medium containing 2% serum and 0.65% agar every 4 or 5 days at 38.5 or 37°, respectively.

To transfect with DEAE-Dextran, the medium was aspirated from the monolayers and cells were washed twice with 2 ml of Tris-buffered saline (0.05 *M* Tris, pH 7.4, and 0.1 *M* NaCl). One-half milliliter of a solution of 500 μg/ml of DEAE-Dextran (Pharmacia, MW 2×10^6) and viral DNA in Tris-buffered saline was layered onto the monolayer and incubated for 20 min at room temperature. After incubation, the monolayers were washed twice with 2 ml of Tris-buffered saline and overlaid with 5 ml of 0.65% agar containing Dulbecco's modified medium and 2% serum.

For transfection with $(Ca)_3(PO_4)_2$, medium was removed from the monolayer and 0.5 ml of a solution containing viral DNA, 12 μg of salmon sperm DNA, 0.125 *M* $CaCl_2$ in Hepes-buffered saline (8.0 g/liter of NaCl, 0.37 g/liter of KCl, 0.125 g/liter of $Na_2HPO_4 \cdot 2H_2O$, 1.0 g/liter of dextrose, 5.0 g/liter of *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, final pH 7.05) was added. After incubation for 20 min at room temperature, 5 ml of conditioned medium (that originally collected from the dish) was added to the dish and this was incubated at 37° in a CO_2 incubator for 4 hr. After 4 hr, the medium was removed and replaced with 5 ml of 0.65% agar in Dulbecco's modified medium with 2% serum.

Electron microscopy. DNA was prepared for observation in the electron microscope (Philips 201) by the aqueous technique of Davis *et al.* (1971). DNA molecules were photographed with 35 mm film, enlarged approximately 10-fold, and printed. The length distribution of DNA was determined by measuring prints with a numonics measuring device.

RESULTS

To prepare Ad5 DNA–protein complexes also use for transfection, we avoided treating virions with chemical agents that might irreversibly denature proteins. The procedure described by Robinson *et al.* (1973) for the isolation of the Ad5 DNA–protein complex involved extracting the disrupted virions with chloroform isoamyl alcohol. This step can be avoided by resolving the virion protein from viral DNA by sedimentation in a sucrose gradient containing 4 *M* guanidinium chloride. Ad5 virions were therefore prepared from infected cells by standard procedures including two sedimentation equilibrium bandings in CsCl gradients. Purified virus was dialyzed into low salt buffer and diluted with an equal volume of 8 *M* guanidinium chloride. After a 5-min incubation on ice, the mixture was layered onto a continuous gradient of 5–20% sucrose also containing 4 *M* guanidinium chloride and sedimented for 17 hr at 4°. An example of such a gradient is shown in Fig. 1. Two peaks are resolved when the gradient is scanned for optical density at 260 nm and some absorbing material remains at the top of the gradient. The faster sedimenting peak has a ratio of absorption at 260 nm/280 nm of about 1.75 while for the second peak this ratio is less than 0.8. Pronase-digested and phenol-extracted ^{32}P-labeled Ad5 DNA cosediments with the fastest sedimenting OD peak, consistent with this being linear viral DNA. The slower sedimenting peak contains capsid proteins.

The cosedimentation of viral DNA from virions disrupted with 4 *M* guanidinium chloride and linear ^{32}P-labeled marker DNA suggested that the conformation of the former was linear. Circular viral DNA would be expected to sediment 10% faster than the linear marker (Hershey *et al.*, 1963). When an aliquot of this fraction was

diluted and immediately prepared for examination in the electron microscope, approximately 96% of the DNA was seen as intact linear duplex DNA; the remaining 4% was circular. However, when the fractions containing viral DNA in Fig. 1 were pooled, dialyzed against 0.1 *M* NaCl, 0.01 *M* Tris, pH 7.9 and 0.001 *M* EDTA and then mounted for visualization in the electron microscope, over 65% of the viral DNA was circular or in more complicated configurations. All of the configurations seen are consistent with the binding or aggregation of proteins attached to the termini of the viral DNA (Table 1). Pronase digestion of these samples resulted in linear duplex DNA.

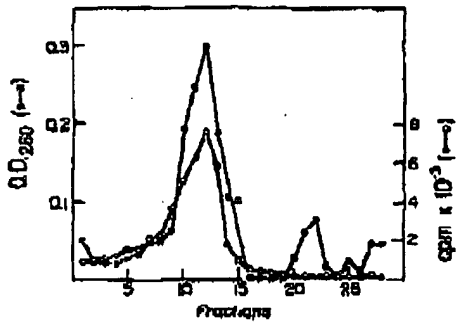

FIG. 1. Sedimentation of Ad5 DNA–protein complex and ^{32}P-labeled viral DNA. Ad5 virions prepared as described under Methods and Materials were dialyzed against 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.9. Virus (0.15 ml; ~1 OD_{260} units of virus) and 6 *M* guanidinium chloride (0.15 ml) were mixed and left at 4° for 5 min. ^{32}P-labeled Ad5 DNA (sp act 5 × 10^5 cpm/μg) was added and the solution was layered onto a 12-ml gradient (SW41) of 5–20% sucrose containing a uniform concentration of 6 *M* guanidinium chloride and 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.9. After centrifugation for 16 hr at 4° and 50,000 rpm, the gradient was collected in 0.4-ml fractions. The absorption of each fraction at 260 nm and the Cerenkov counts of 20 μl of each fraction were determined.

In an attempt to fractionate the various complex forms from one another, an aliquot of the dialyzed protein–DNA complex was layered onto a 1.4% agarose gel and electrophoresis was carried out for 8 hr. As shown in Fig. 2, while viral DNA prepared by Pronase digestion readily enters the gel during electrophoresis, the DNA–protein complex is almost quantitatively retained on the surface of the gel. If the protein–DNA complex is cleaved by a restriction endonuclease, both terminal fragments are retained at the surface of the gel while all internal fragments migrate into the gel at a rate expected from their molecular weights (see below). Digestion of either intact DNA–protein complexes or restriction endonuclease cleavage products of them with Pronase eliminates the anoma-

TABLE 1

DISTRIBUTION OF STRUCTURES IN Ad5 DNA–PROTEIN COMPLEX[a]

Structures	Monomer		Dimer		Lariat		More complex forms
	Linears	Circles	Linears	Circles	Monomer (circle–linear)	Dimer circle–monomer linear	
Number	34	9	5	2	2	1	4
Length (cm)	24.2 ± 1.0	23.8 ± 1.5	47.6 ± 1.3	44.5 ± 2.1	45.0 ± 3.8	69.0	—
Mass[b]	34	9	10	4	4	3	14
Percentage by mass	35	13	15	6	6	4	21

[a] Ad5 DNA–protein complex prepared as described in the legend to Fig. 1 and in the text, was diluted in 5 *M* $NH_4C_2H_3O_2$ and mounted for electron microscopy as described under Methods and Materials. Typical areas of a grid were photographed and printed. All structures observed on the prints were classified and measured. A lariat is a circular DNA molecule with a linear segment projecting from one point on the circumference of the circle.

[b] One unit is equal to monomer length Ad5 DNA.

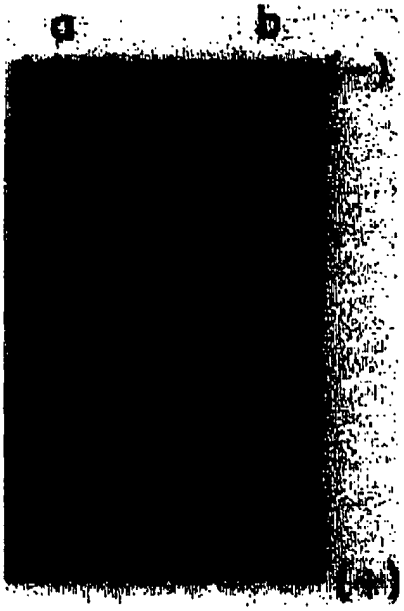

FIG. 2. Gel electrophoresis of Ad5 DNA–protein complex. Ad5 DNA–protein complex pooled from gradients as shown in Fig. 1 was dialyzed against 0.1 *M* NaCl, 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.8. One-tenth microgram of the Ad5 DNA–protein complex was added to each of two 100-μl reaction mixes of 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.8. To one reaction mix, 10 μg of Pronase was added and both were incubated at 37° for 10 min. After incubation, both reaction mixes were layered onto 1.4% agarose gels and a potential gradient at 80 V was applied for 14 hr. Following electrophoresis, the gels were stained with 0.5 μg/ml of ethidium bromide and photographed. Only the top third of the gels is shown; however, no additional bands were observed. The band of DNA in gel (b) comigrates with unit length Ad5 DNA. Gel (a) is the Ad5 DNA–complex and gel (b) is the Ad5 DNA–complex digested with Pronase.

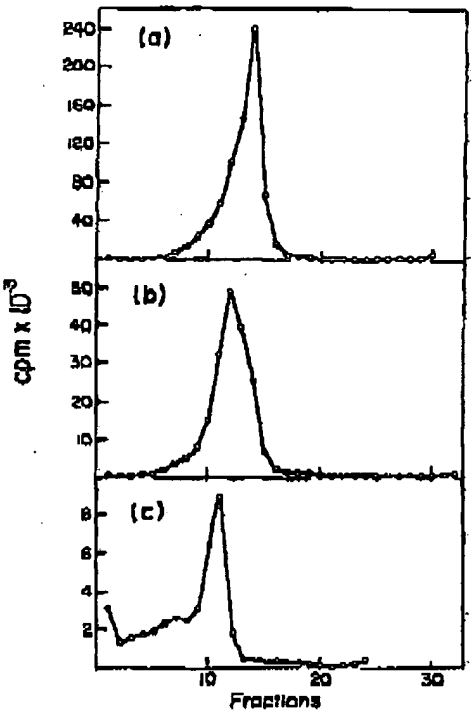

FIG. 3. Sedimentation of ^{32}P-labeled Ad5 DNA–protein complex in 4 *M* guanidinium chloride gradients. (a) ^{32}P-labeled Ad5 virus was prepared with a specific activity of 5×10^5 cpm/μg. ^{32}P-labeled viral DNA (8×10^4 cpm) or 0.2 ml of virus was mixed with an equal volume of 8 *M* guanidinium chloride and centrifuged in an SW41 gradient as described in Fig. 1. The gradient was centrifuged for 8.5 hr at 39,000 rpm and 0.4-ml fractions were collected. b) Peak fraction, No. 14, of the gradient shown in (a) was diluted with 0.9 ml of water and layered directly onto a duplicate 5–20% sucrose gradient containing 4 *M* guanidinium chloride and centrifuged for 9 hr at 39,000 rpm and 0.4-ml fractions were collected. (c) Two adjacent fractions from the gradient shown in panel a and similar fractions from a duplicate gradient run in parallel were pooled and dialyzed against 0.1 *M* NaCl, 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.8. One-half milliliter of the dialyzed sample was mixed with 0.5 ml of 4 *M* guanidinium chloride and sedimented for 5 hr at 39,000 rpm onto a 5–20% sucrose gradient containing 4 *M* guanidinium chloride. 0.5-ml fractions were collected and Cerenkov counted.

lous retention at the surface of the gel. Therefore we conclude that although 35% of the protein-DNA complex fraction did not form circular or complex configurations (Table 1) after dialysis, most of the DNA in the sample contained protein attached to at least one terminus.

In order to characterize further the Ad5 DNA–protein complex, ^{32}P-labeled virions

were prepared, disrupted with 4 *M* guanidinium chloride, and sedimented through a 5–20% sucrose gradient as described previously. A single peak of ^{32}P-labeled DNA was observed at a position expected for linear DNA (Fig. 3a). To resolve viral DNA further from free protein, the peak fraction of this gradient (fraction 14) was diluted with buffer and sedimented into a second identical gradient (Fig. 3b). The remaining fractions of the first gradient (Ad5 complex I) and the pooled fractions of the resedimented ^{32}P-labeled DNA (Ad5 complex II) were dialyzed at 4° against saline buffer (0.1 *M* NaCl, 0.01 *M* Tris, 0.001 *M* EDTA, pH 7.9). These dialyzed samples will be referred to as Ad5 complex I and Ad5 complex II, respectively. When the configuration of viral DNA in both preparations was scored after mounting for the electron microscope, the proportion of circular and more complicated forms was very similar (Table 2). In these samples approximately 50% of the viral DNA molecules were linear duplexes and the ratio of linear to circular forms was approximately 3. In contrast, when material from the peak fraction of the first gradient was mounted directly by dilution from the 4 *M* guanidinium chloride sucrose solution, 89% of the DNA was linear duplex and the ratio of linear to circular forms was 20 (Table 2, Ad5 fraction 14). Hence, the viral DNA-protein complex is linear when released from the virions by treatment with 4 *M* guanidinium chloride and the circular and more complicated configurations form during dialysis into a saline solution. This association of protein at the termini of the viral DNA is not simply reversible, as diluting the Ad5 complex I into 4 *M* guanidinium chloride and then mounting it for observation with the electron microscope did not change the ratio of linear to circular forms (Table 2). In addition, when an aliquot of the ^{32}P-labeled Ad5 complex I was mixed with an equal volume of 8 *M* guanidinium chloride and then sedimented through a 5–20% sucrose gradient containing 4 *M* guanidinium chloride, approximately 50% of the ^{32}P-labeled DNA sedimented faster than monomer length linear DNA (Fig. 3c). Thus, the association of the protein at the ter-

TABLE 2

CONFORMATIONS OF ^{32}P-LABELED Ad5 DNA–PROTEIN COMPLEX PREPARATIONS[a]

Preparation	Monomer		Oligomer			
	Linear	Circles	2 units	3 units	>4 units	Other
Ad5 complex I	60	21	6	13	4	2
	61.0%	14.4%	6.5%	28.7%	10.9%	
Ad5 complex II	69	24	10	11	2	1
	51.0%	18.8%	11.5%	20.0%	4.6%	
Ad5 (fraction 14)	120	8	4	—	—	
	89.0%	4.5%	6.0%			
Ad5 complex I	128	36	ND	ND	ND	
	78.0%	22.0%				
Ad5 complex I (4 *M* guanidinium chloride)	93	31	ND	ND	ND	
	75.6%	24.4%				

[a] ^{32}P-labeled Ad5 DNA–protein complexes were prepared as shown in Fig. 3 and dialyzed against saline buffer. Aliquots of these samples were diluted 10-fold and mounted for electron microscopy as described under Methods and Materials. In all cases, the classification of different structures was done by scoring while viewing the sample in the electron microscope. Ad5 (fraction 14): An aliquot of fraction 14 of the gradient shown in Fig. 3a was diluted 1/10 directly from the gradient fraction containing 4 *M* guanidinium chloride and immediately mounted for electron microscopy. Ad5 DNA complex I (4 *M* guanidinium chloride): An aliquot of Ad5 DNA complex I was mixed with an equal volume of 8 *M* guanidinium chloride and incubated for 10 min at room temperature. This sample was then mounted in a fashion identical to Ad5 (fraction 14). The control preparation of Ad5 complex I was mounted in an identical fashion but without dilution with 8 *M* guanidinium chloride. Only the number of linear and circular monomer length molecules were scored in both of these preparations.

mini of the Ad5 complex during dialysis into a saline buffer is not readily reversible.

Although approximately 50% of the DNA in Ad5 complex I remained linear after dialysis, over 88% of the ^{32}P-labeled viral DNA was retained at the surface of a 1.4% agarose gel. As Pronase-digested Ad5 complex I quantitatively entered the gel, the retention at the gel surface must be due to the presence of protein associated with the ^{32}P-labeled DNA. Similar results were obtained with the Ad5 complex II sample that had been twice sedimented through the 4 *M* guanidinium chloride sucrose gradients. To show that the protein responsible for the retention at the surface of the gel was associated with the termini of the genome, ^{32}P-labeled Ad5 complex I was digested with the restriction endonuclease *Eco* R1 and half of the reaction mix was layered directly onto a 1.4% agarose gel. The other half of the reaction mix was digested briefly with Pronase before layering onto the gel. All of the Pronase-digested ^{32}P-labeled DNA entered the gel and resolved into the three expected fragments for *Eco* R1 cleavage of Ad5 DNA. However, 94 and 95% of the A and B fragments, respectively, of the *Eco* R1 digestion of the ^{32}P-labeled Ad5 complex I were retained at the top of the gel, while the internal *Eco* R1 fragment C entered the gel unabated (Fig. 4). Similar experiments using the restriction endonuclease *Hpa* 1 which cleaves 4 and 12% from the left and right termini, respectively, of the genome gave identical results (Sambrook *et al.*, 1975); that is, only the two terminal fragments of the Ad5 complex are prevented from entering the gel.

As 50% of the viral DNA in the Ad5 complex I preparation was linear when scored by the electron microscope while over 90% of both terminal fragments were retained at the surface of the 1.4% agarose gel, it seemed highly probable that linear DNA had protein attached to both of its termini. To test this possibility further, we examined the susceptibility of the ^{32}P-labeled Ad5 complex I to degradation by the *adenosine triphosphate-dependent deoxyribonuclease* from *Hemophilus influenzae* Rd (Friedman and Smith, 1972a,b). This ATP-dependent DNase does not degrade circular double-strand DNA but readily cleaves linear duplex DNA (Friedman and Smith, 1972a,b). Hence the ATP-dependent DNase requires a free end on duplex DNA to initiate degradation of the molecule. Either short 3′ or 5′ single-strand tails at the termini of the duplex DNA do not significantly inhibit the rate of digestion (Friedman and Smith, 1972a,b). The ATP-dependent DNase (a generous gift of Drs. M. Mann and H. Smith) was tested for specificity under our reaction conditions on a mixture of linear and circular phage λ DNA. Circularization of λ DNA is due to the annealing of a 12 base long sequence at one terminus of the DNA to a complementary sequence at the other terminus (Wu and Taylor, 1971). A reaction mix containing 0.5 μg of both circular and linear λ DNA was digested with 0.75 units of the ATP-dependent DNase for 15 min at 37° and the products resolved on an 1.4% agarose gel with markers run in parallel. Linear λ DNA was completely degraded while hydrogen-bonded circular λ DNA was resistant to digestion (data not shown).

The susceptibility of Ad5 complex I to degradation by the ATP-dependent DNase was examined in two ways. Linear SV40 duplex DNA, produced by cleavage with the *Hpa* II restriction endonuclease (Sharp *et al.*, 1973), and Ad5 complex I DNA were each added at a concentration of 3 μg/ml. A 10-μl aliquot of the reaction mix was diluted and mounted for observation with the electron microscope (Fig. 5a). To the remainder of the reaction mix, 0.75 units of the ATP-dependent DNase was added and incubated for 15 min at 37°. Again, 10 μl of the reaction mix after incubation were mounted for observation in the electron microscope (Fig. 5b). A perfunctory survey of Figs. 5a and b shows that the linear SV40 DNA was degraded while linear and circular Ad5 length DNA was resistant to digestion. Histograms of the DNA length in the reaction mix before and after the addition of enzyme are shown in Fig. 6. The length measurements confirm the initial observations. While linear Ad5

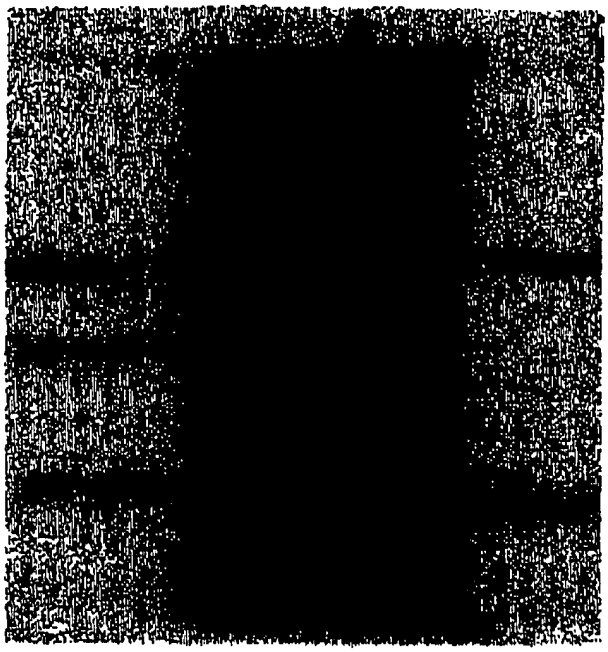

FIG. 4. Gel electrophoresis of *Eco* R1 cleavage products of ^{32}P-labeled Ad5 DNA–protein complex. ^{32}P-labeled Ad5 DNA–protein complex prepared as described in legend to Fig. 3 was dialyzed against two 1-liter volumes of 0.1 *M* NaCl, 0.01 *M* Tris, and 0.001 *M* EDTA, pH 7.9. One-tenth milliliter of the ^{32}P-labeled DNA complex was diluted with 0.1 ml of buffer, giving a final salt concentration of 0.05 *M* NaCl, 0.01 *M* Tris, pH 7.9, and 0.01 *M* $MgCl_2$. After heating to 37° for 10 min, *Eco* R1 endonuclease was added and incubation continued for 10 min. After incubation, 0.05 ml was transferred to each of two reaction mixes and 1 μl of 20-mg/ml Pronase was added. After incubation for 10 min at 37°, both samples were mixed with bromophenol blue and layered onto 0.8 × 12-cm 1.4% agarose gel. Electrophoresis was carried out for 6 hr at 30 V. The two gels were stained and photographed and then sliced into 1-mm slices for counting. The Cerenkov counts per minute indicated beside the bands were calculated by correcting for background and are summations of the values for three or more adjacent slices. Three bands of ^{32}P-labeled DNA, *Eco* R1 fragments A (78.7), B (18.0), and C (7.3), were resolved from the sample digested with Pronase, gel (a). The proportion of ^{32}P counts per minute in each band is that expected for the known molecular weights of the *Eco* R1 cleavage fragments of Ad5 DNA (Sambrook *et al.*, 1975). Over 90% of both end fragments A and B are retained at the surface of gel (b) when the *Eco* R1 digestion products of the Ad5 DNA–protein complex are directly layered onto gel.

DNA was resistant to digestion, essentially all linear SV40 was cleaved into fragments smaller than half-length.

To quantitate better the fraction of ^{32}P-labeled Ad5 complex I resistant to digestion by the ATP-dependent DNase, the products of similar reaction mixes were analyzed by agarose gel electrophoresis (Fig. 7). Gel (a) shows the results of electrophoresis of a reaction mix containing ^{32}P-labeled Ad5 complex I; the ^{32}P-label is retained at the top of the gel. In the second sample resolved in gel (b), 0.2 μg of unlabeled Ad5 DNA was mixed with 0.15 μg of the ^{32}P-labeled complex I; again the ^{32}P-labeled DNA was retained at the surface of the gel while the added unlabeled Ad5 DNA entered the gel unaffected. A duplicate reaction mix containing both 0.15 μg of ^{32}P-labeled Ad5 complex I and unlabeled

FIG. 5. Digestion of Ad5 DNA–protein complex by ATP-dependent DNase. ^{32}P-labeled Ad5 complex I and linear SV40 DNA produced by cleavage with the restriction endonuclease *Hpa* II were mixed and mounted for visualization in the electron microscope. A typical field with both linear SV40 DNA and Ad5 DNA is shown in (a). After digestion of the remainder of this sample with the ATP-dependent DNase, an aliquot was mounted for electron microscopy (b). Note that linear SV40 does not survive the digestion, while linear Ad5 DNA and a lariat dimer have not been degraded by the nuclease. A length histogram for DNA presence before and after digestion is shown in Fig. 6. For details of conditions for digestion by the ATP-dependent DNase, see legend to Fig. 6.

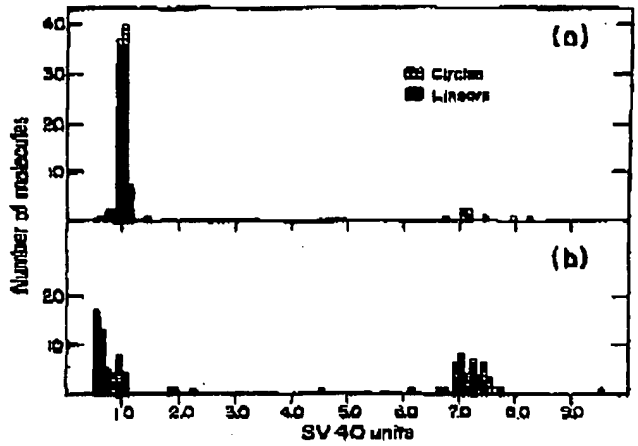

FIG. 6. Digestion of Ad5 complex DNA by ATP-dependent DNase. ^{32}P-labeled Ad5 complex I was diluted to a concentration of 3 μg/ml in 0.10 *M* Tris, pH 8.0, 0.01 *M* $MgCl_2$, 0.33 m*M* ATP, and 0.001 *M* DTT. Linear SV40 duplex DNA produced by digestion of form I DNA (Hirt, 1967) with the restriction endonuclease *Hin* II (Sharp *et al.*, 1973) was added to a concentration of 3 μg/ml. A 10-μl sample of this mix was diluted in 5 *M* NH_4 $C_2H_3O_2$ and mounted for observation in the electron microscope (Davis *et al.*, 1971). Then 0.75 units of ATP-dependent DNase was added to the remainder (0.05 ml) and incubated for 15 min at 37°. Following incubation, 10 μl of the reaction mix was again mounted for electron microscopy. Grids of both digested and nondigested samples were photographed on 35-mm film, enlarged, and printed. The length of all DNA segments longer than 0.5 SV40 units (0.85 cm) on several typical prints were measured and normalized to unit length SV40. However, only monomer length circular or linear Ad5 DNA were plotted. Circular DNA is denoted by open squares (□) and linear DNA segments by filled squares (■). Ad5 DNA has a molecular weight of 24×10^6 daltons and would be expected to be 7.03 times longer than SV40 DNA (3.4×10^6 daltons). Histogram (a) shows length distribution before digestion while histogram (b) shows DNA length after digestion. Note many more grid areas were measured for data shown in (b).

Ad5 DNA was digested with the ATP-dependent DNase and layered onto gel (c). Digestion by the ATP-dependent DNase completely cleaved the free Ad5 DNA but did not cleave the ^{32}P-labeled Ad5 complex I. Quantitation of the percentage of the ^{32}P-labeled complex resistant to the ATP-dependent DNase is given by comparing the amount of ^{32}P DNA migrating as intact DNA in gels (d) and (e). In the reaction mix layered onto gel (d), 0.15 μg of ^{32}P-labeled Ad5 complex I and 0.2 μg of unlabeled Ad5 DNA were digested for 15 min with 0.75 units of the ATP-dependent DNase and then for 20 min with 20 μg of Pronase. The reaction mix layered onto gel (e) contained 0.15 μg of the ^{32}P-labeled Ad5 complex I digested only with 20 μg of pronase. The band migrating as intact Ad5 DNA in gel (d) contains 80% of the ^{32}P-labeled DNA that is present in the equivalent band in gel (e) (duplicate experiment gave 81%). We conclude from these experiments that the Ad5 DNA–protein complex is resistant to the ATP-dependent DNase and that digestion with Pronase removes the agent preventing the action of the nuclease.

The infectivity of purified DNA from any adenovirus serotype has been very low. Using the technique of precipitation of DNA with $(Ca)_3(PO_4)_2$, Graham *et al.* (1973) observed an efficiency of infection of 10–20 PFU/μg for Ad5 DNA. It is interesting to note that the efficiency of transformation of mammalian cells by adenovirus DNA is approximately the same as that for infectivity (Graham and Van der Eb, 1973a,b). To test whether the viral DNA–protein complex described above (see Fig. 1) had a higher efficiency of infection than viral DNA, aliquots of the sample were

FIG. 7. ^{32}P-labeled Ad5 DNA-protein complex resistance to ATP-dependent DNase digestion. Five reaction mixes (0.05 ml) containing 3 μg/ml of ^{32}P-labeled Ad5 complex I were prepared in a buffer of 0.16 *M* Tris, pH 8.0, 0.35 m*M* ATP, 0.01 *M* $MgCl_2$, and 0.001 *M* DTT. The five reaction mixes were ultimately layered onto the gels (a–e) and resolved by electrophoresis for 4 hr at 70 V. After staining, the gels were photographed and then sliced into 1-mm sections and Cerenkov counted. The history of each reaction mix is: (a) simply incubated at 37° for 15 min; (b) 0.2 μg of Ad5 DNA was added before incubation at 37° for 5 min; (c) 0.2 μg of Ad5 DNA and 0.75 units of ATP-dependent DNase were added and incubated for 15 min at 37°; (d) 0.75 units of ATP-dependent DNase was added and incubated for 15 min at 37°; immediately thereafter the reaction mix was augmented with 20 μg of Pronase and incubation at 37° was continued for 10 min; (e) ^{32}P-labeled complex was incubated for 10 min at 37° and then 20 μg of Pronase was added and incubation was continued for 10 min. Only the top halves of the gels are shown, but there were no additional bands observed in the gels. The position of intact Ad5 DNA is marked by the arrows. The bands marked by the arrow in gels (d) and (e) contained 1240 and 1680 cpm, respectively.

mixed with 12 μg of salmon sperm carrier DNA and were used in the $(Ca)_3(PO_4)_2$ protocol for transfection (Graham and Van der Eb, 1973a,b). Our Ca technique was previously tested using SV40 DNA and an efficiency of 10^6 PFU/μg was observed on a monkey cell line, CV-1. The results of these experiments are given in Table 9 for the preparation of DNA-protein complex described in Figs. 1 and 2. This series of experiments spans a period of more than a month. The highest efficiency of transfection observed with Pronase-digested, phenol-extracted Ad5 DNA was 20–25 PFU/μg. In many cases, no plaques developed when 1 μg or more of viral DNA was added to the cells. A wide range of infectivity was observed with the DNA–protein complex, 47–7820 PFU/μg. However, in any given set of experiments, the DNA–protein complex was 10^2-fold more infectious than free DNA. This was also observed with other preparations of Ad5 DNA and Ad5 DNA–protein complexes. That the infectivity of the DNA–protein complex was not due to contaminating virus was shown by the absence of plaques when no $CaCl_2$ was added to the DNA infectivity assay and by the susceptibility of the infectious material to digestion by DNase. Ad5 virus forms plaques with 100% efficiency in the transfection assay without $CaCl_2$ and was not susceptible to DNase digestion. The reduction in plaques after addition of Ad5 antiserum was probably due to an inhibition of the transfection assay by the added protein; a precipitate forms in the $(Ca)_3(PO_4)_2$ solution after addition of the antiserum and similar reductions in efficiency of transfection were observed with Ad5 antiserum and SV40 DNA. We conclude that the Ad5 protein–DNA complex has a higher efficiency of transfection than free viral DNA.

TABLE 3

TRANSFECTION BY Ad5 DNA–PROTEIN COMPLEX*

	Sample	Concentration of DNA (μg)	$CaCl_2$	Plaques/dish	PFU/μg
(a)	Ad5 DNA–protein complex	1.0	+	59, 47	59–47
	Ad5 DNA–protein complex	1.0	−	0, 0	0, 0
	Ad5 DNA–protein complex	0.1	+	16, 24	160–240
	Ad5 DNA	1.0	+	0, 0	0
	Ad5DNA + Ad5 DNA–protein complex	0.5 + 0.5	+	19, 22	38–44
(b)	Ad5 DNA–protein complex	1.0	−	0, 0	−
	Ad5 DNA	0.25	+	0, 0	−
	Ad5 DNA	0.50	+	13, 10	26–20
	Ad5 DNA	1.0	+	8, 11	8–11
	Ad5 DNA–protein complex	0.1	+	421, 350	4510–3500
	Ad5 DNA–protein complex	1.0	+	TM, TM	−
		0.0	+	0, 0	−
(c)	Ad5 DNA	1.0	+	5, 5	5–5
	Ad5 DNA–protein complex	0.1	+	25, 46	250–460
	Ad5 DNA–protein complex	1.0	+	446, 426	446–426
(d)	Ad5 DNA	1.0	+	1, 2	1–2
	Ad5 DNA–protein complex	0.1	+	14, 14	140
	Ad5 DNA–protein complex	0.5	+	140, 180	280–320
(e)	Ad5 DNA–protein complex	0.1	+	52, 130	520–1300
	Ad5 DNA–protein complex	0.1 + Ad5 antiserum	+	0, 3	0, 30
	Ad5 DNA–protein complex	1.0	−	0, 0	0, 0
	Ad5 DNA–protein complex	0.1 + DNase	+	0, 0	0, 0
	Ad5 DNA–protein complex	0.1 incubated 37°	+	28, 35	280–350
(f)	Ad5 DNA–protein complex	1.0	+	TM, TM	−
	Ad5 DNA–protein complex	0.1	+	782, 680	7820–6800
(g)	Ad5 DNA–protein complex	1.0	+	TM, TM	−
	Ad5 DNA–protein complex	0.1	+	167, 173	1670–1730
	Ad5 DNA–protein complex	0.1	+	418, 403	4180–4030

* Transfection was performed as described under Methods and Materials. The results are grouped (a–g) as experiments carried out on a single day. Ad5 DNA–protein complex used in experiments (a)–(e) was the preparation described in Fig. 1 and characterized in Fig. 2. The Ad5 DNA–protein complex sample used in (f) and (g) was prepared in a similar fashion and the infectivity of this sample is also sensitive to DNase digestion and dependent on the addition of $CaCl_2$ (data not shown). DNase treatment consisted of incubating complex in 0.1 *M* NaCl, 0.01 *M* Tris, pH 8.0, 0.01 *M* $MgCl_2$ with 10 μg/ml of pancreatic DNase for 30 min at 37°. Equivalent incubation of complex alone in this buffer did not decrease infectivity. This digestion condition degrades duplex DNA into small oligonucleotides. A 0.01-ml aliquot of Ad5 antiserum (purchased from Flow Laboratories) was added to a 0.4-ml solution of complex (0.1 *M* NaCl, 0.01 *M* Tris, pH 8.0, and 0.001 *M* EDTA, pH 8.0). This solution was incubated for 30 min at 37° and then used for transfection. Addition of an equivalent amount of antiserum to Ad5 virus under these conditions neutralizes over 10^9 PFU of virus.

DISCUSSION

Ad5 DNA–protein complexes were prepared by disrupting virions with 4 *M* guanidinium chloride and resolving the viral DNA from slower sedimenting proteins by centrifugation in a sucrose gradient containing 4 *M* guanidinium chloride. Under these conditions, the released viral DNA sediments at a rate expected for linear duplex molecules and, when mounted for observation with the electron microscope, 90% of the Ad5 DNA is observed to be linear. However, after dialysis into saline buffer, approximately 50% of the Ad5 DNA recovered from such gradients form circular or more complicated configurations. All of these configurations are consistent with the aggregation of termini of the viral

DNA. Throughout this paper and in publications from other laboratories, this association of the termini of the DNA is normally credited to the presence of protein at that position (Robinson and Bellett, 1974; Brown *et al.*, 1975). The basis for this suggestion is that digestion with Pronase converts the Ad5 DNA preparation to monomer length linear duplex DNA. Although it is highly likely that a protein is involved, it has not yet been chemically demonstrated. With this reservation in mind, we will continue to describe the characteristics of Ad5 DNA prepared with this protocol in terms of a DNA–protein complex.

Robinson *et al.* (1973) first isolated adenovirus DNA–protein complexes by disrupting virions with 4 *M* guanidinium chloride and then extracting with chloroform. Such DNA–protein complexes sediment as circular viral DNA in 4 *M* guanidinium chloride sucrose gradients. Our modification of their procedure consisted of eliminating the chloroform extraction. Ad5 DNA–protein complexes sediment as linear duplex DNA in the sucrose gradient if the chloroform extraction is avoided. Circular and more complicated configurations form during subsequent dialysis; such dialysis renders these configurations resistant to disassociation by 4 *M* guanidinium chloride. If the termini of the Ad5 DNA–protein complex had aggregated during the chloroform extraction step in the procedure of Robinson *et al.* (1973), the two results would have been consistent. Nevertheless, the observation that only 50% of the Ad5 DNA–protein complexes seem to irreversibly aggregate into complicated forms suggests that the process might not be due to the simple affinity of two proteins for each other or of one protein for the two termini of the viral DNA. In fact, it is possible that the association of the termini of the Ad5 DNA–protein complex *in vitro* is due to protein denaturation and that *in vivo* the genome is linear. The experiments described here and those already published are inconclusive on this point.

The exclusion of adenovirus DNA–protein complex from entering an agarose–acrylamide composite gel has also been reported by Brown *et al.* (1975). Here, Ad2 DNA–protein complexes were isolated by disassociating virions with 0.05% SDS. In addition, Brown *et al.* (1975) also showed that the terminal fragments produced by *Eco* R1 digestion were retained at the surface of the gel while the internal fragments comigrated with marker viral DNA.

Although 50% of the ^{32}P-labeled Ad5 DNA–protein complex was linear after dialysis into saline buffer, over 90% of the ^{32}P-labeled DNA was retained at the surface of an agarose gel during electrophoresis. *Eco* R1 digestion of Ad5 DNA produces three fragments, A, B, and C. The two largest fragments A and B are from the left and right ends, respectively. Over 90% of both the A and B fragments remained at the gel surface during electrophoresis when ^{32}P-labeled Ad5 DNA–protein complex was cleaved by *Eco* R1 digestion and layered onto an agarose gel. Pronase digestion of this sample before electrophoresis allowed both fragments to migrate normally. Thus, most of the linear Ad5 DNA–protein complex must have protein attached to both termini. The susceptibility of the Ad5 DNA–protein complex to degradation by the ATP-dependent DNase from *Hemophilus influenzae* Rd supports this conclusion. This DNase requires a free terminus, probably for a site of entry, to degrade duplex DNA (Friedman and Smith, 1972a,b). Hydrogen-bonded circular phage λ DNA is resistant to digestion under conditions where linear λ DNA is completely degraded. When the preparation of the Ad5 DNA–protein complex was digested with the ATP-dependent DNase, both linear and circular genome length DNA were resistant. Under similar conditions, free Ad5 DNA was completely degraded. Proteins attached to both termini of the viral DNA therefore protect the linear DNA from degradation. This conclusion is based on two assumptions: First, the DNase would use either terminus to commence degradation if it were unblocked, and second, that Ad5 DNA–protein complexes with a linear conformation as seen by the electron microscope are also linear in aqueous solution.

Interestingly, the resistance of the Ad5

DNA–protein complex to degradation by the ATP-dependent DNase also provides one possible explanation for the increased infectivity of the complex when compared to free viral DNA. Hence, the protein at the termini of the viral DNA may protect it from degradation by similar exonucleases *in vivo*. The stimulus for this suggestion comes from the reduction of phage T7 DNA transfection efficiency by the equivalent ATP-dependent DNase in *E. coli*, the recBC exonuclease (Benzinger *et al.*, 1975). The genome of T7 is a linear duplex and transfection of an recBC⁺ *E. coli* gives approximately 20 PFU/μg of T7 DNA. The level of efficiency of transfection increases to 50-fold when an recBC⁻ *E. coli* host is used (Benzinger *et al.*, 1975). However, as initiation of both adenovirus 5 DNA replication (Sussenbach *et al.*, 1972) and RNA transcription (Sharp *et al.*, 1974) probably occurs at or near both termini of the genome, the viral proteins attached to ends of the genome could have several functions.

The efficiency of transfection of the Ad5 DNA–protein complex varied from a low of 60 to a maximum of 7820 PFU/μg. In all of the experiments, the efficiency of transfection by the complex was significantly higher than that observed with free viral DNA. We have not been able to identify the cause of the variable efficiency. If the efficiency of transfection can be reproducibly maintained in the rang of 10^4 PFU/μg, it may be possible to modify the viral DNA *in vitro* and the propagate mutants by transfection.

Note added in proof. Several phages of *Bacillus subtilis* yield DNA–protein complexes similar to those described in this manuscript for adenovirus 5 [Ortin, J., Vinuela, E., Salas, M., and Vasquez, C. (1971). *Nature New Biol.* 234, 275–277]. Hirokawa (1972) (*Proc. Nat. Acad. Sci. USA* 69, 1555–1558) reported that the efficiency of transfection of DNA from Φ29, a *B. subtilis* phage, was sensitive to protease digestion. Recently a temperature-sensitive mutant of Φ29 was shown also to be temperature sensitive for efficiency of transfection [Yanofsky, S., Kawamura, F., and Ito, J. (1975). *Nature* 253, 80–83].

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NOTES

Enhanced Infectivity of Adenovirus Type 2 DNA and a DNA-Protein Complex

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The infectivity of adenovirus type 2 DNA and a DNA-protein complex was studied in 293 cells, a human embryonic kidney cell line transformed by sheared adenovirus type 5 DNA, and in human KB cells. Adenovirus type 2 DNA was more infectious (up to about 40-fold) in 293 cells than in KB cells, whereas a DNA-protein complex (prepared by a rapid procedure) had about the same infectivity in both cell lines. These data may mean that a factor present in 293 cells (perhaps a viral-coded protein) enhances the infectivity of free viral DNA. The infectivity of DNA and the DNA-protein complex was increased up to fivefold by brief treatment of cell monolayers with 25% dimethyl sulfoxide after transfection. Under these conditions, (i) the infectivity of native adenovirus type 2 DNA ranged from 400 to 1,300 PFU/μg of DNA in 293 cells and from about 9 to 14 PFU/μg of DNA in KB cells, and (ii) the infectivity of the DNA-protein complex was 6×10^3 to 2×10^4 PFU/μg in 293 cells and 1.4×10^3 to 1.6×10^4 PFU/μg in KB cells.

The infectivity of human adenovirus DNA was first demonstrated by Nicolson and McAllister (10), who observed a typical adenovirus-type cytopathic effect when human embryo kidney cells were treated with adenovirus type 1 (Ad1) DNA in the presence of DEAE-dextran (12). No reproducible relationship was found between DNA concentration and cytopathic effect. A reproducible technique for assaying adenovirus type 5 (Ad5) DNA infectivity was developed subsequently by Graham and van der Eb (4). DNA was co-precipitated with calcium phosphate, and infectivity was quantitated by plaque assay on human KB cells. The infectivity of Ad5 DNA was low (about 20 PFU/μg) compared with other viral DNAs, such as simian virus 40 DNA (10^6 PFU/μg [8]) and herpesvirus type 1 DNA (10^3 PFU/μg [5, 15]). Recently, Sharp et al. (13) reported that the efficiency of transfection by Ad5 DNA could be increased approximately 100-fold with a viral DNA-protein complex (18); however, the infectivity of the DNA-protein complex was highly variable. It was recently reported that the infectivity of herpesvirus type 1 DNA could be increased by treatment of cell monolayers with dimethyl sulfoxide (Me_2SO) after transfection with the calcium phosphate technique (16). In this report, we have determined the infectivity of adenovirus type 2 (Ad2) DNA and a DNA-protein complex in human KB cells and 293 cells, a human embryo kidney cell line transformed by sheared Ad5 DNA. Me_2SO treatment after transfection increased the infectivity of free DNA as well as that of the DNA-protein complex. We describe a modification of the method of Robinson et al. (18) that yielded preparations of DNA-protein complex with a reproducible infectivity of about 10^4 PFU/μg.

The 293 cell line is a human embryonic kidney cell line transformed by sheared Ad5 DNA that was established by Graham et al. (3). This cell line yields plaques with Ad2 and Ad5 up to 4 days earlier than do other established human cell lines, with about the same efficiency as that observed in KB cells and in HeLa cells (F. Graham, personal communication; our unpublished data). We have been using this cell line to assay the infectivity of in vitro-constructed Ad2 mutant DNA. We have compared the infectivity of Ad2 DNA in the presence and absence of Me_2SO in 293 cells. In typical experiments, Ad2 (strain 38-2, plaque 6, free from adenovirus-associated viruses) DNA extracted from virions (6) by the standard protease-sodium dodecyl sulfate-phenol method (7) yielded from about 150 to 400 PFU/μg (different DNA preparations in different experiments) on 293 cells (Table 1). Treatment with 25% Me_2SO in *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered sa-

TABLE 1. *Infectivity of native Ad2 DNA in 293 cells and effect of Me_2SO*[a]

Expt	DNA concn μg/dish	No. of plaques/dish		PFU/μg	
		Without Me_2SO	With Me_2SO	Without Me_2SO	With Me_2SO
1	0.1	40, 32, 41	117, 85, 139	400, 320, 410	1,170, 850, 1,390
	0.2	84, 73, 91	233, 227, 209	420, 366, 455	1,165, 1,825, 1,045
	0.5	158, 180, 195	—[b]	316, 360, 390	—
2	0.2	26, 29	84, 83	130, 145	420, 415
	0.5	75, 72	—	150, 144	—

[a] Each experiment was carried out with a different DNA preparation on different days. The transfection assays were carried out by the calcium phosphate technique of Graham and van der Eb (4) with minor modifications. Briefly, 293 cells growing in Eagle minimal essential medium containing 10% horse serum were removed from monolayers with a mixture of 0.05% trypsin and 0.025% EDTA in phosphate-buffered saline, diluted with growth medium, centrifuged, resuspended in growth medium, and seeded (2×10^6 cells) in 60-mm Falcon petri dishes. Cell sheets were used on the following day for transfection. Calcium phosphate precipitation of viral DNA was performed at room temperature for 15 to 20 min in *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered saline (4) containing 20 μg of phenol-chloroform-extracted salmon sperm DNA per ml (Sigma Chemical Co.) and 125 mM $CaCl_2$. The calcium phosphate precipitate (0.5-ml suspension) was added to each cell monolayer after removal of growth medium. After 20 min at room temperature, 5 ml of fresh growth medium was added, and cultures were incubated at 37°C for 4 h. The medium was removed, and plates were overlaid with 10 ml of agar overlay medium or treated with Me_2SO followed by the addition of agar overlay. The overlay medium consisted of minimal essential medium in 0.9% agar (Difco) supplemented with 5% horse serum and 0.1% yeast extract for 293 cells or with 5% horse serum and 5% chicken serum for KB cells. A second overlay (5 ml) containing 0.0022% neutral red was added on day 7 for 293 cells or on day 10 for KB cells. Plaques were counted 2 days later. Treatment with Me_2SO was carried out essentially as described by Stow and Wilkie (16). Cell monolayers at 4 h after transfection were washed with minimal essential medium (2 or 5% serum) and treated with 1 ml of sterile 25% Me_2SO in *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered saline at room temperature for 4 min. Me_2SO was removed, and cell sheets were washed with 3 ml of minimal essential medium containing serum, followed immediately by the addition of agar overlay as described above.

[b] —, Not tested.

line for 4 min at 4 h after the addition of the calcium phosphate DNA precipitate yielded from 400 to 1,300 PFU/μg. In other experiments, plates treated with Me_2SO always yielded more plaques than parallel plates receiving no Me_2SO.

Recently, Sharp and co-workers (13) have reported that the DNA-protein complex prepared by disruption of Ad2 virions with 4 M guanidinium chloride followed by sedimentation in a sucrose density gradient containing 4 M guanidinium chloride was about 100-fold more infectious than free DNA when assayed in human embryonic kidney cells. As noted by these authors, a wide variability in the number of plaques (47 to 7,820 PFU/μg) was observed. We prepared the DNA-protein complex by a modification of the method of Robinson et al. (18). Virions were disrupted with 4 M guanidinium chloride in the presence of mercaptoethanol, and the DNA-protein complex was purified on Sepharose 4B. The entire operation was carried out in 2 to 3 h at 0 to 4°C to avoid possible denaturation of the complex. The DNA-protein complex prepared from ^{32}P-labeled Ad2 by this procedure yielded a single homogeneous peak (Fig. 1) with a ratio of absorbancy at 260 nm to that at 280 nm greater than 1.9 (indistinguishable from that of free DNA).

The DNA-protein complex prepared as described above was further characterized, inasmuch as it was prepared in a somewhat different manner than previously described. When the DNA-protein complex was cleaved with restriction endonuclease *Eco*RI and subjected to electrophoresis through agarose gels, the terminal A and C fragments (9) were retained on the gel surface (Fig. 2, lane 3). When digested with Pronase, the fragments readily entered the gel (Fig. 2, lane 4). Digestion of the DNA-protein complex with other restriction endonucleases, including *Bam*HI, *Sal* I, and *Xba*, followed by electrophoresis on agarose gels, gave identical results (data not shown). Similar observations have been made with Ad2 and Ad5 DNA-protein complexes prepared by different methods (2, 11, 13). Therefore, the DNA-protein complex used in the present study contained the protein attached to both terminal fragments of Ad2 DNA.

The infectivity of the DNA-protein complex in 293 cells and the effect of Me_2SO treatment are given in Table 2. The DNA-protein complex yielded an average of from 3×10^3 to 1.6×10^4 PFU/μg in three different experiments. Me_2SO treatment increased the specific infectivity from two- to fivefold. The infectivity of the DNA-protein complex was completely abolished by treatment with DNase; moreover, no plaques resulted in the absence of precipitation with

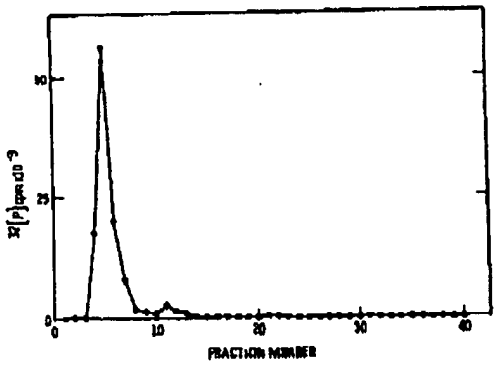

FIG. 1. *Isolation of an Ad2 DNA-protein complex by chromatography on Sepharose 4B. $CsCl$-purified Ad2 (6) was diluted to an absorbancy at 260 nm of 8 to 10 (^{32}P-labeled virus mixed with unlabeled virus) and dialyzed against two changes of 500 volumes of 10 mM Tris-hydrochloride (pH 8.1) containing 1 mM EDTA and 2 mM β-mercaptoethanol at 0°C for 6 h. An equal volume of 8 M guanidinium chloride (Schwarz/Mann) in Tris-hydrochloride–EDTA–mercaptoethanol was added, and the suspension was incubated at 0°C for 10 min and loaded onto a Sepharose 4B (Pharmacia) column (2 by 26 cm) equilibrated with 4 M guanidinium chloride in Tris-hydrochloride–EDTA–mercaptoethanol at 4°C. Fractions were collected at a flow rate of about 18 ml/h. Fractions containing the DNA-protein complex were detected by measurement of radioactivity or absorbancy at 260 nm, pooled, and dialyzed against 10 mM Tris-hydrochloride (pH 7.6) containing 1 mM EDTA and 2 mM mercaptoethanol. The DNA-protein complex (30 to 50 μg/ml) was stored at 0°C up to 2 months for transfection studies without appreciable loss in infectivity. The concentration of DNA in preparations of the DNA-protein complex was determined from the specific radioactivity or from the absorbancy at 260 nm (the absorption spectrum of the DNA-protein complex is indistinguishable from that of DNA purified by treatment with pronase-sodium dodecyl sulfate-phenol).*

calcium phosphate (data not shown). Thus, Me_2SO had a stimulatory effect on the infectivity of both Ad2 DNA and the DNA-protein complex in 293 cells.

KB cells are used extensively as a host cell for the study of Ad2 replication. We therefore studied the effect of Me_2SO on the infectivity of Ad2 DNA and the DNA-protein complex in these cells. Ad2 DNA yielded an average of 5 and 8 PFU/μg in KB cells in two experiments (Table 3). Similar values have been obtained by Graham and van der Eb (4) with Ad5 DNA. When Me_2SO treatment was used, the infectivity increased to an average of 9 and 14 PFU/μg of Ad2 DNA (Table 3). Thus, the infectivity of native DNA in KB cells is about 40-fold less than that in 293 cells (Table 1).

The effects of Me_2SO on the infectivity of the DNA-protein complex in KB cells are given in Table 4. The DNA-protein complex gave an average of 1.2×10^6 PFU/μg. Me_2SO treatment increased infectivity to about 1.6×10^4 PFU/μg. Although KB cells were not as sensitive as 293 cells to Me_2SO treatment, a definite enhancement of plaque formation in Me_2SO-treated monolayers was always observed. It is noteworthy that the infectivity of the DNA-protein complex is similar in 293 cells (Table 2) and KB cells (Table 4), whereas the infectivity of native DNA was much higher in 293 cells than in KB cells.

In this study we have determined the infectivity of Ad2 DNA and a DNA-protein complex in both 293 cells and KB cells and shown that infectivity is enhanced by treatment with Me_2SO. The infectivity of herpesvirus type 1 DNA was increased about 100-fold by Me_2SO treatment (16). Although we did not observe as dramatic an increase in the infectivity of Ad2 DNA and DNA-protein complex, a consistent enhancement of infectivity compared with that in untreated monolayers (up to about fivefold) was observed. The mechanism by which Me_2SO

TABLE 2. *Infectivity of DNA-protein complex in 293 cells and effect of Me_2SO*[a]

Expt	DNA concn μg/dish	No. of plaques/dish		PFU/μg	
		Without Me_2SO	With Me_2SO	Without Me_2SO	With Me_2SO
1	0.01	—[b]	111, 134, 118	—	11,100, 13,400, 11,800
	0.02	47, 44, 53	142, 109, 116	2,350, 2,200, 2,650	7,100, 5,420, 5,800
	0.05	165, 173, 167	—	3,300, 3,460, 3,340	—
2	0.01	—	175, 177, 195	—	17,500, 17,700, 15,950
	0.02	165, 138, 160	339, 338, 319	8,250, 6,900, 8,000	16,950, 16,900, 15,950
	0.05	266, 211, 250	—	5,320, 4,220, 5,000	—
3	0.01	164, 81, 100	213, 183, 220	16,400, 8,100, 11,000	21,300, 18,300, 22,000

[a] Experiments 2 and 3 were carried out with the same DNA-protein complex preparation; experiment 1 was conducted with a different preparation.

[b] —, Not tested.

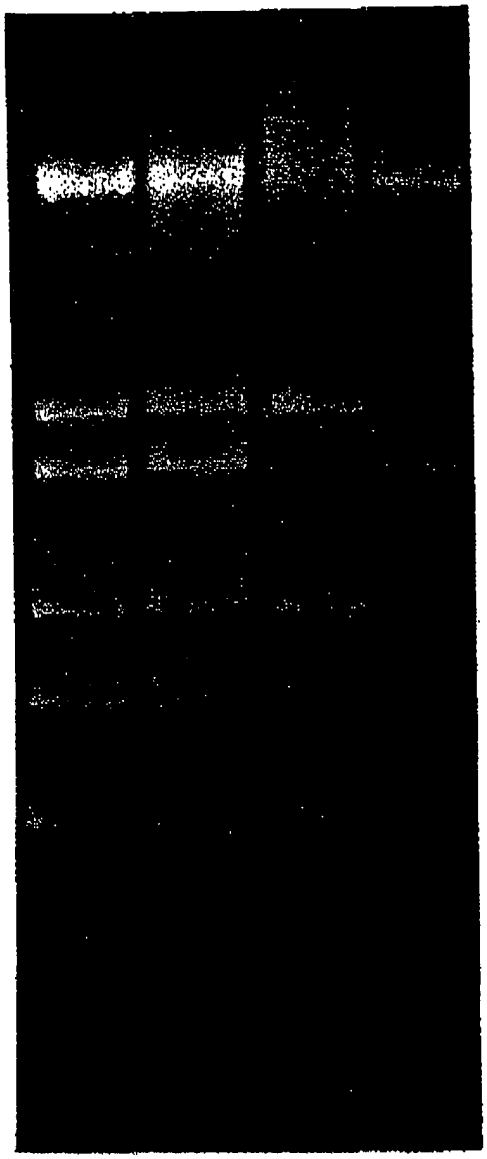

FIG. 2. *Gel electrophoresis of Ad2 DNA and the DNA-protein complex after cleavage with restriction endonuclease EcoRI. Electrophoresis was carried out in 1.4% agarose slab gel at 40 V for 14 h as described previously (14). (1) EcoRI-digested Ad2 DNA. (2) EcoRI-digested DNA-protein complex after incubation with 500 μg of Pronase per ml at 37°C for 1 h. (3) EcoRI-digested DNA-protein complex. (4) Fragments retained on the surface of the gel after electrophoresis of EcoRI-digested DNA-protein complex were eluted, incubated with 500 μg of Pronase per ml at 37°C for 1 h, and subjected to electrophoresis as described above.*

TABLE 3. *Infectivity of Ad2 DNA on KB cells and effect of Me₂SO*[a]

Expt	No. of plaques/dish	
	Without Me_2SO	With Me_2SO
1	5, 6, 12 (8)	17, 13, 11 (14)
2	5, 7, 4 (5)	8, 12, 6 (9)

[a] These experiments were carried out at a DNA concentration of 1 μg/dish. A single DNA preparation was used in both experiments. Numbers in parentheses are averages.

increases the infectivity of viral DNA is not known.

Transfection of KB cells with Ad2 DNA gave about 5 to 8 PFU/μg. Similar results were reported for Ad5 DNA (3). On the other hand, about 150 to 400 PFU/μg of DNA were observed in 293 cells. Graham, who developed the 293 cell line, has also observed in 10- to 50-fold stimulation of infectivity of Ad5 DNA in 293 cells compared with that in other established human cell lines (quoted in reference 3).

It has been shown that the Ad5 DNA-protein complex (13) and the Ad2 DNA-protein complex (present study) are substantially more infectious than DNA purified by the standard protease-sodium dodecyl sulfate-phenol method (7). The 293 cell line retains about 14% of the left end and about 5% of the right end of the Ad5 genome (R. Weinman, personal communication) and expresses only the left end as mRNA (3). Graham et al. (3) reported evidence that some viral-induced polypeptides are synthesized by 293 cells. These polypeptides may play some role in enhancing the infectivity of viral DNA. The protein bound covalently to adenovirus DNA (11, 17) appears necessary for the increased infectivity of viral DNA (13; present study). It is therefore possible that this protein may be synthesized by 293 cells and thereby may account for the enhanced infectivity of free Ad2 DNA. Other possible explanations include (i) lower levels of nucleases in 293 cells, by analogy with *Escherichia coli* strains deficient in *recBC* nucleases that are responsible for the enhanced infectivity of bacteriophage DNAs (1), and (ii) more efficient uptake of viral DNA by 293 cells than by KB cells.

In the present study, we have shown that the infectivity of the Ad2 DNA-protein complex ranged from about 6×10^3 to 2×10^4 PFU/μg in 293 and KB cells. DNA-protein complex prepa-

TABLE 4. *Effect of Me$_2$SO on infectivity of the Ad2 DNA-protein complex in KB cells*[a]

Expt	No. of plaques/dish		PFU/μg	
	Without Me$_2$SO	With Me$_2$SO	Without Me$_2$SO	With Me$_2$SO
1	218, 268, 261	271, 312, 342	10,900, 13,400, 13,050	13,550, 15,600, 17,100
2	215, 262, 250	322, 315, 316	10,750, 13,100, 12,500	16,100, 15,750, 15,800

[a] These experiments were carried out at a DNA concentration of 0.02 μg/dish.

rations used over a period of 4 months exhibited only about a fivefold variation in infectivity. Others (13) have reported a wider variation (47 to 7,820 PFU/μg) in the infectivity of an Ad5 DNA-protein complex. Our method for the preparation of the DNA-protein complex is somewhat different and may be responsible for the increased reproducibility of transfection. The improved transfection technique described here should be very useful for manipulation of the adenovirus genome in vitro.

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